Mast Cells Increase Vascular Permeability by Heparin-Initiated Bradykinin Formation In Vivo

Chris Oschatz,[1,2] Coen Maas,[1,2] Bernd Lecher,[4] Thomas Jansen,[1,2,5] Jenny Björkqvist,[1,2] Thomas Tradler,[6] Reinhard Sedlmeier,[7] Peter Burfeind,[8] Sven Cichon,[9,10,11] Sven Hammerschmidt,[12] Werner Müller-Esterl,[13] Walter A. Wuillemin,[14,15] Gunnar Nilsson,[3] and Thomas Renné[1,2,*]

[1]Clinical Chemistry, Department of Molecular Medicine and Surgery
[2]Center of Molecular Medicine
[3]Clinical Immunology and Allergy Unit, Department of Medicine
Karolinska Institutet, 17176 Stockholm, Sweden
[4]mfd diagnostics GmbH, 55234 Wendelsheim, Germany
[5]Department of Medicine II, University Medical Center Mainz, 55131 Mainz, Germany
[6]Scil Proteins GmbH, 06120 Halle, Germany
[7]Ingenium Pharmaceuticals GmbH, 82152 Martinsried, Germany
[8]Institute of Human Genetics, University of Göttingen, 37073 Göttingen, Germany
[9]Department of Genomics, Life & Brain Center
[10]Institute of Human Genetics
University of Bonn, 53105 Bonn, Germany
[11]Institute for Neuroscience and Medicine, Research Center Jülich, 52425 Jülich, Germany
[12]Department of Genetics of Microorganisms, Interfaculty Institute of Genetics and Functional Genomics, University of Greifswald, 17487 Greifswald, Germany
[13]Institute for Biochemistry II, University of Frankfurt, 60590 Frankfurt, Germany
[14]Division of Hematology and Central Hematology Laboratory, Luzerner Kantonsspital, CH-6000 Luzern, Switzerland
[15]University of Bern, CH-3010 Bern, Switzerland
*Correspondence: thomas@renne.net
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SUMMARY

Activated mast cells trigger edema in allergic and inflammatory disease. We report a paracrine mechanism by which mast cell-released heparin increases vascular permeability in vivo. Heparin activated the protease factor XII, which initiates bradykinin formation in plasma. Targeting factor XII or kinin B2 receptors abolished heparin-triggered leukocyte-endothelium adhesion and interfered with a mast cell-driven drop in blood pressure in rodents. Intravital laser scanning microscopy and tracer measurements showed heparin-driven fluid extravasation in mouse skin microvessels. Ablation of factor XII or kinin B2 receptors abolished heparin-induced skin edema and protected mice from allergen-activated mast cell-driven leakage. In contrast, heparin and activated mast cells induced excessive edema in mice deficient in the major inhibitor of factor XII, C1 esterase inhibitor. Allergen exposure triggered edema attacks in hereditary angioedema patients, lacking C1 esterase inhibitor. The data indicate that heparin-initiated bradykinin formation plays a fundamental role in mast cell-mediated diseases.

INTRODUCTION

Mast cells are multifunctional effector cells of the immune system. Their role in initiating allergic and anaphylactic reactions

and their activation during certain types of parasitic infection are well recognized (Marshall, 2004). However, mast cells appear to make a much broader contribution to other aspects of immune regulation and are crucial in host defense responses (Galli et al., 2008). These cells produce an array of vasoactive mediators and proinflammatory substances that are either preformed granule-associated mediators, newly generated lipid mediators, or belong to a wide variety of cytokines and chemokines (Kalesnikoff and Galli, 2008).

A well-established concept is that mast cell (MC) mediators, particularly the vasoactive amines histamine and serotonin, increase capillary leakage and may rapidly produce edema. Histamine receptor antagonists are therapeutically used to treat edema formation and allergic reactions associated with aberrant mast cell activity, e.g., in asthmatic disease (Theoharides and Kalogeromitros, 2006). MC secretory granules also contain highly sulfated polysaccharides, with heparin as a major constituent. This glycosaminoglycan, which is synthesized exclusively by MC, has a crucial role as a depot for various mediators and for the morphology of the granules (Forsberg et al., 1999; Humphries et al., 1999). Although heparin is one of the most studied molecules, its physiological function in vivo has yet to be determined.

Formation of the vasoactive and proinflammatory peptide hormone bradykinin (BK) in vitro can be initiated by a variety of polyanionic surfaces, such as kaolin, glass, ellagic acid, certain polymers, nucleotides, sulfatides, polyphosphates, misfolded proteins, and some types of collagen or glycosaminoglycans (Cochrane and Griffin, 1982; Maas et al., 2011). Generation of BK involves the plasma contact system proteins factor XII (FXII), plasma prekallikrein, high-molecular-weight kininogen (HK), and C1 esterase inhibitor (C1INH). Binding to negatively
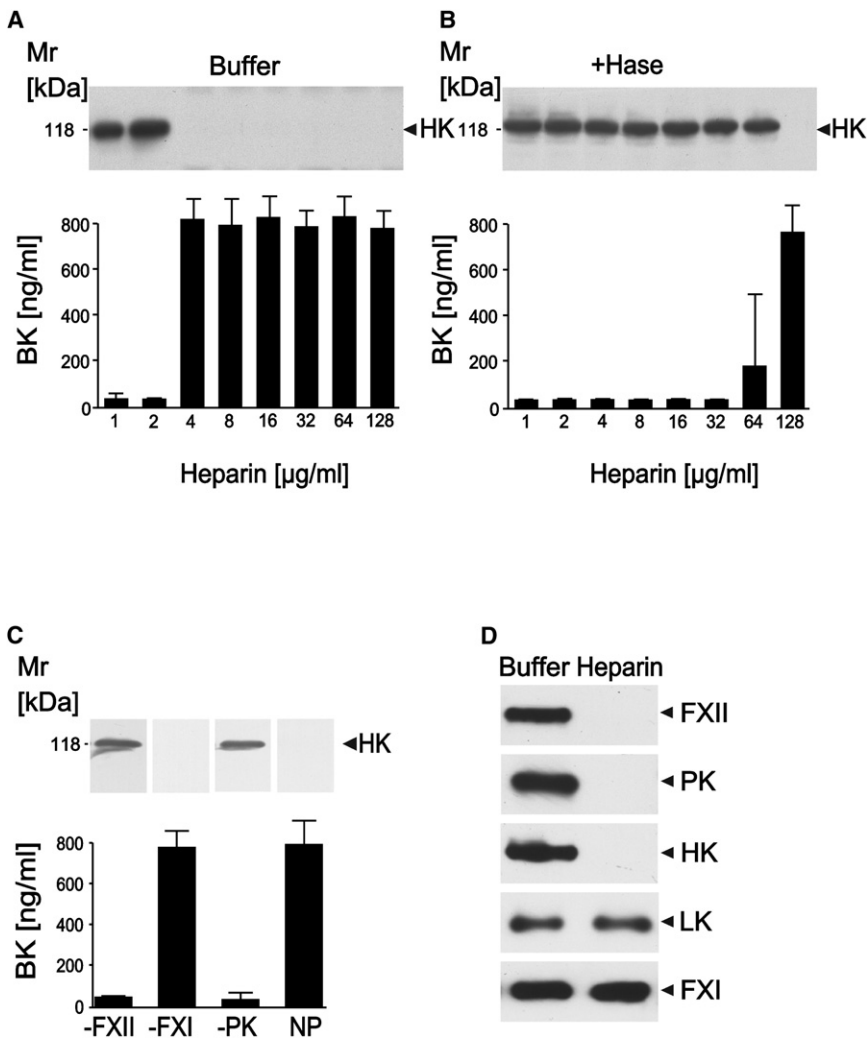
Figure 1. MC-Derived Heparin Initiates Contact System-Mediated BK Formation

Human plasma was incubated for 30 min at 37°C with MC-derived glycosaminoglycans and analyzed for single-chain HK by immunoblotting and for BK formation by ELISA (means ± SD, n = 8).

(A) Glycosaminoglycans at ≥4 μg/ml initiated plasma HK cleavage and BK formation.

(B) MC-derived glycosaminoglycans were incubated with Hase prior to addition of plasma.

(C) Plasma deficient in FXII, FXI, or PK and normal plasma (NP) was incubated with 20 μg/ml MC-heparin for 30 min and analyzed for HK cleavage and BK liberation.

(D) Buffer-treated and heparin-treated (Heparin) plasma was probed for FXII, PK, single-chain HK, LK, and FXI zymogen, respectively.

By using human plasma and endothelial cells, pharmacological inhibitors in rats, genetically altered mice, and patients with inherited C1INH deficiency, we show that heparin-triggered BK formation is a critical component of MC-evoked edema with implications for HAE. Targeting heparin-initiated BK formation may represent a new strategy to protect against defective barrier function attributed to MC activation in allergic, anaphylactic, atopic, and inflammatory disease.

RESULTS

Mast Cell-Heparin Activates the Contact System in Plasma

We isolated high-molecular heparin (500–750 kDa) from rat peritoneal mast cells and analyzed the potency of the glycosaminoglycans for BK generation. Human plasma was incubated with increasing amounts of heparin and analyzed for HK processing and BK formation by immunoblotting and ELISA, respectively. Antibodies against the BK sequence in HK revealed that ≥4 μg/ml heparin initiated complete plasma HK cleavage (Figure 1A, top). Consistently, BK was high (>780 ng/ml) in these samples but low in those treated with buffer (37 ± 22 ng/ml) or with ≤ 2 μg/ml heparin (<35 ng/ml), which was not sufficient to initiate HK processing (Figure 1A, bar graph). As a control, we incubated MC-derived glycosaminoglycans with heparinase (Hase) before adding them to plasma. The enzyme specifically digests heparin. Hase treatment largely abolished BK formation, triggered by activated MC-released material. HK conversion started at >30-fold higher concentrations of the digested (≥128 μg/ml, Figure 1B) versus nontreated glycosaminoglycans, indicating that heparin accounts for most of the BK-forming activity in MC-released material. Heparin induces the reciprocal activation of FXII and PK in ice-cold plasma (Hojima et al., 1984). Consistent with this observation, heparin failed to initiate HK cleavage and BK production in FXII- or PK-deficient plasma at physiological temperature (Figure 1C). In contrast, deficiency of the FXII

charged surfaces induces a conformational change in FXII zymogen, resulting in a small amount of active FXII (FXIIa). FXIIa cleaves prekallikrein to generate active plasma kallikrein (PK), which in turn reciprocally activates additional FXII. This amplification loop generates sufficient PK activity to liberate BK from HK by limited proteolysis (Müller and Renné, 2008). BK binding to its cognate B2 receptor (B2R) activates various intracellular signaling pathways that dilate vessels, induce chemotaxis of neutrophils, and increase vascular permeability and fluid efflux (Leeb-Lundberg et al., 2005). Excessive BK generation resulting from hereditary deficiency of functional C1INH (the major plasma inhibitor of FXIIa, PK, and several complement proteases) causes a life-threatening swelling disorder, hereditary angioedema (HAE). HAE patients experience recurrent attacks of swelling, but the stimuli that trigger these periodic episodes of excessive vascular leakage are poorly defined (Davis, 2008; Zuraw, 2008). In allergic disease, BK is generated and contributes to increased vascular permeability (Kaplan and Ghebrehiwet, 2010; Proud and Kaplan, 1988). In vitro, heparin liberates BK by triggering contact system activation (Hojima et al., 1984; Brunnée et al., 1997; Noga et al., 1999). However, it is not known whether heparin functions in mast cell-triggered vascular effects in vivo.
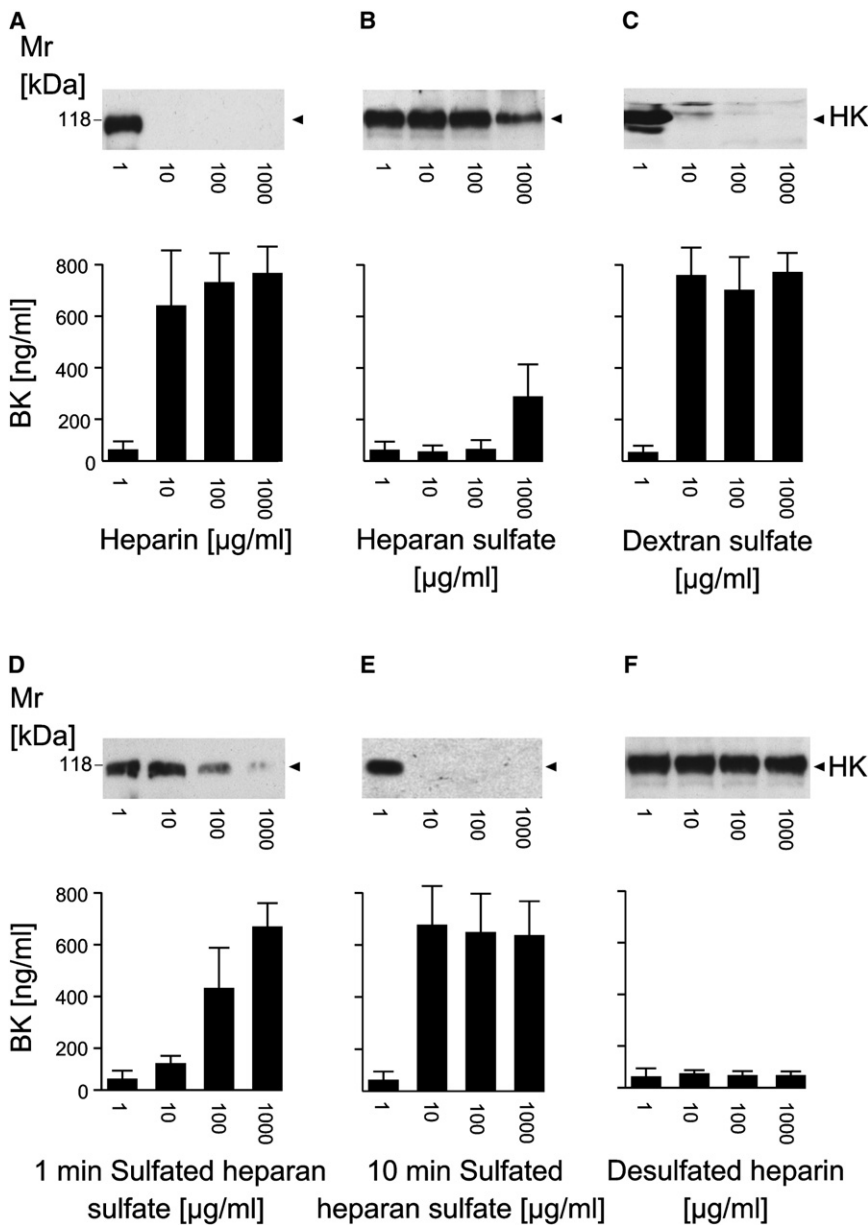
Figure 2. MC-Heparin-Induced BK Formation Is Dependent on the Negative Charge Density of the Polyanion

Human plasma samples were supplemented with 1–1000 μg/ml heparin (A), heparan sulfate (B), dextran sulfate (C), or heparan sulfate sulfated for 1 min (D) or 10 min (E) and desulfated heparin (F), incubated for 30 min at 37°C, and analyzed for HK cleavage and BK formation by immunoblotting and ELISA. Means ± SD, n = 8 are plotted.

amounts of heparin, heparan sulfate, and the synthetic polysaccharide dextran sulfate (a potent FXII activator; Johne et al., 2006), respectively (Figures 2A–2C). Heparin and dextran sulfate at ≥ 10 μg/ml each triggered complete conversion of plasma HK. In contrast, heparan sulfate up to 100 μg/ml did not induce HK cleavage, and high concentrations (1000 μg/ml) of the glycosaminoglycan induced only minor HK cleavage (36% of total plasma HK, corresponding to 260 ng/ml BK). To demonstrate that negative charge density, rather than a defined structure of the polysaccharide, is responsible for the enhanced BK-generating potency of heparin as compared to heparan sulfate, we sulfated heparan sulfate with sulfuric acid and carbodiimide. This method does not cause depolymerization of the polysaccharide. Hypersulfation increased the BK-forming activity of heparan sulfate (Figure 2D). After 10 min sulfation, the activity of treated heparan sulfate was similar to heparin (compare Figures 2E versus 2A). In contrast, chemical desulfation of heparin with N,O-bis(trimethylsilyl)acetamide largely abolished its BK-forming activity (Figure 2F). Together, the data show that heparin initiates BK formation in plasma via activation of the contact system.

Mast Cell-Heparin Competes with HK for Binding to Cells

BK formation is tightly regulated and HK binding to the cell surface heparan sulfate-type proteoglycans protects the BK-precursor from proteolysis (Renné et al., 2005b). Because both heparin and heparan sulfate bind to the cell-binding site in HK (Leeb-Lundberg et al., 2005), we reasoned that heparin might detach cell-bound HK from heparan sulfates as a prerequisite for efficient BK formation. To test this hypothesis, we incubated EA.hy926 endothelial cells with 60 nM radiolabeled, uncleaved HK, removed unbound HK, and added HK-deficient plasma supplemented with heparin (0.05–200 μg/ml). This experimental
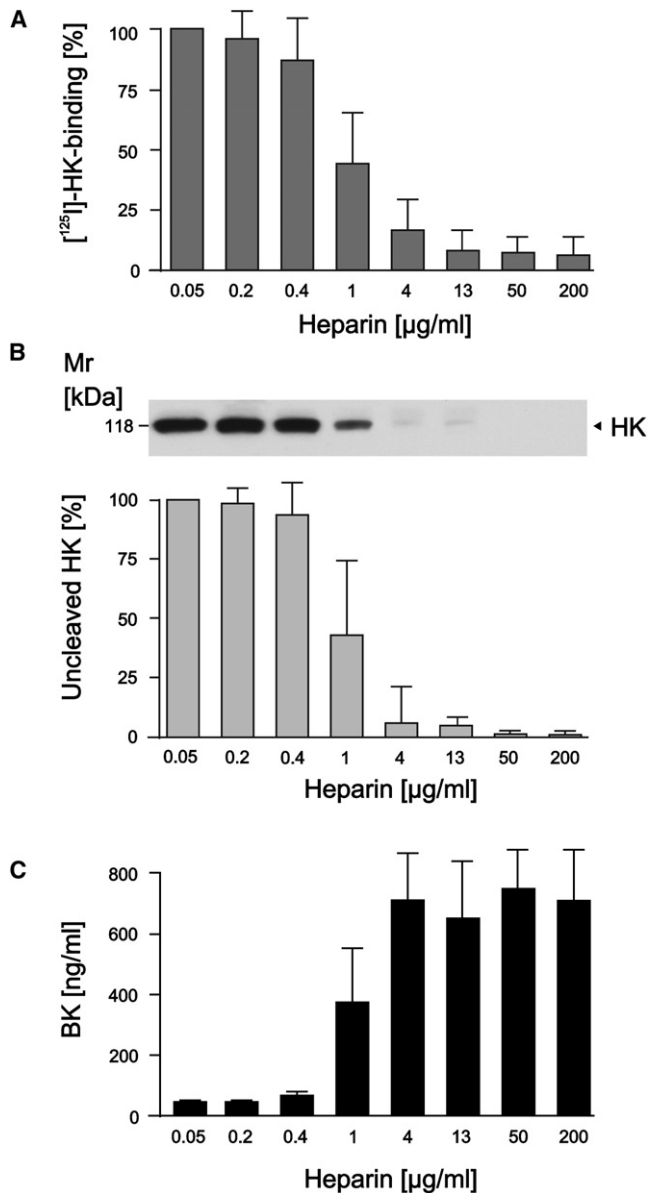
substrate in the intrinsic coagulation pathway, factor XI (FXI), did not interfere with HK processing (Figure 1C, lane 2). Heparin added to normal plasma induced the contact system cascade with sequential proteolytic activation of FXII, PK, and HK, but not of low-molecular-weight kininogen (LK, a kininogen gene splice variant that does not bind PK) or FXI (Figure 1D; Figure S1A available online). Efficient contact system activation depends on negative charge density and the size of the FXII-binding surface (Müller and Renné, 2008). We compared heparin and heparan sulfate for their ability to release BK from HK. Both polysaccharides have similar backbones consisting of alternating iduronic acids and glucosamines, but differ in charge (heparin has an average of 2.7 sulfate groups per typical disaccharide unit, whereas heparan sulfate has an average of 1.0; Capila and Linhardt, 2002). Human plasma was incubated with increasing

Figure 3. MC-Heparin Detaches HK from Endothelial Cells and Induces BK Release

Uncleaved radiolabeled HK (60 nM) bound to EA.hy926 cells was incubated with 0.05–200 μg/ml heparin in HK-deficient plasma. After incubation for 30 min at 37°C, the samples were analyzed for HK cell binding, HK cleavage, and BK formation.

(A) The cellular [^{125}I]-HK binding capacity was monitored by a γ-counter and normalized for HK binding in the absence of heparin (set at 100%).

(B) Cleavage of HK was analyzed by immunoblotting with antibodies against the BK sequence. The relative amounts of uncleaved HK, determined from immunoblot signal intensities, are given below (untreated control set at 100%).

(C) BK in cell supernatants, determined by ELISA.

Means ± SD of five independent experiments are shown.

design analyzes cell-bound HK in the absence of HK in the fluid phase. After incubation, we monitored cell-bound HK, HK cleavage, and BK formation. Heparin dose dependently reduced HK-cell binding; at ≥4 μg/ml polysaccharide, cell-bound HK

was reduced to <20% of the amounts of cell-bound HK measured in the absence of heparin (set to 100%; Figure 3A). HK detachment from cells was associated with HK cleavage (Figure 3B) and BK formation (Figure 3C). At ≥4 μg/ml heparin, HK was almost completely cleaved (<7% single-chain HK; Figure 3B) and BK was formed concomitantly with HK processing. BK generation started at >0.4 μg/ml and reached maximal concentrations (>700 ng/ml) at ≥4 μg/ml heparin (Figure 3C). We conclude that heparin has a dual function in BK generation: the polysaccharide displaces HK from cells *and* it activates FXII.

Mast Cell-Heparin Activates the Contact System in Rodents

To test heparin for triggering BK generation in rodents, we infused the polysaccharide into the femoral artery of rats and analyzed BK formation by ELISA. Heparin injection greatly increased plasma BK (224 ± 93 ng/ml) over buffer-treated animals (23 ± 16 ng/ml). BK generation was associated with leukocyte adhesion to vessel walls, as shown in limb cross-sections from animals that were sacrificed 30 min after challenge (Figure 4A). Leukocyte-endothelial cell adhesion was restricted to femoral artery vessel walls downstream of the injection site and to the ipsilateral vena femoralis. At some sites, leukocytes had penetrated the endothelial border and invaded subendothelial tissues (Figure 4B). To confirm that BK mediates heparin-triggered leukocyte adhesion, we treated rats with the B2R antagonist Icatibant (Wirth et al., 1991) prior to heparin infusion. Icatibant did not alter heparin-induced BK plasma concentrations (181 ± 72 ng/ml) but almost completely blunted leukocyte adhesion to vessel walls (Figures 4C and 4D). In addition to being a chemoattractant for neutrophils, BK is a potent vasodilator (Leeb-Lundberg et al., 2005). Intra-arterial heparin infusion induced a rapid (within 45 s), pronounced (∼160 to 50 mmHg), and reversible drop in blood pressure. Blood pressure recovered close to baseline within 30 min. Heparin-triggered hypotonic reaction was almost completely blocked by Icatibant (Figure 4E). In contrast to intra-arterial application, subcutaneous or intravenous injection of heparin up to 25 mg/kg bodyweight (bw) did not change blood pressure nor trigger leukocyte adhesion (data not shown). To evaluate the role of heparin-driven BK formation for allergic reactions, we challenged wild-type (WT), FXII-deficient ($F12^{-/-}$), and B2R-deficient ($Bdkrb2^{-/-}$) mice that are defective in BK generation (Pauer et al., 2004) or BK signaling (Borkowski et al., 1995), respectively, in a model of passive systemic anaphylaxis. We injected mice intravenously with anti-DNP IgE and challenged them 24 hr later with an intravenous injection of DNP-human serum albumin (DNP-HSA). WT mice responded with a rapid and transient drop in systemic arterial blood pressure of 57 ± 24 mmHg, whereas allergen-induced hypotonic response was decreased in $F12^{-/-}$ (23 ± 10 mmHg) and $Bdkrb2^{-/-}$ (27 ± 13 mmHg) mice. WT mice pretreated with either FXII-inhibitor PCK (Kleinschnitz et al., 2006) or Icatibant were protected from IgE-allergen (Ag)-mediated drop in blood pressure (25 ± 10 and 29 ± 12 mmHg; Figure 4F). We analyzed for HK cleavage concomitant with BK release in plasma of IgE-Ag-challenged mice. 10 min after antigen infusion, circulating HK was processed and the BK moiety was released from its precursor in
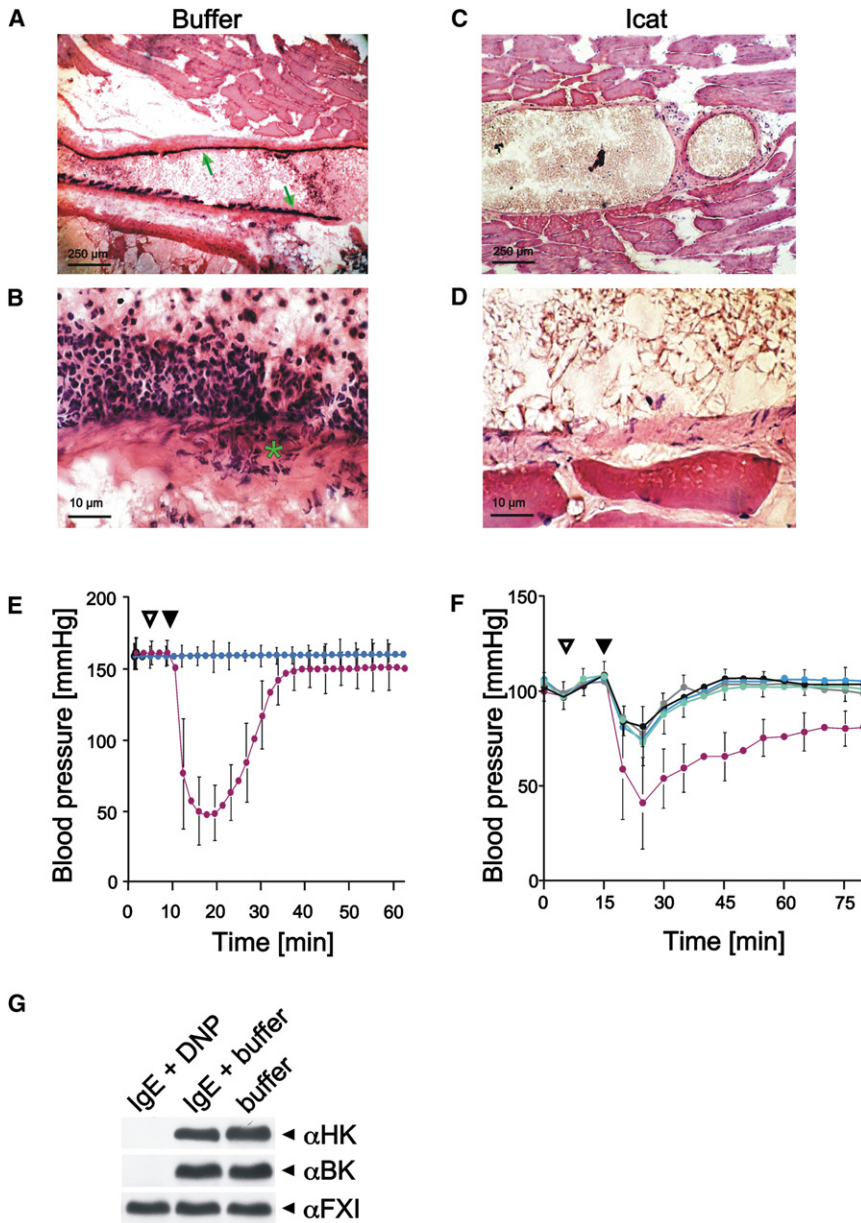
Figure 4. MC-Heparin Induces BK-Driven Leukocytes Infiltration and Hypotension in Rodents

(A–D) Heparin was infused into the left femoral artery of untreated (Buffer) or Icatibant (Icat)-treated rats and the animals were sacrificed 30 min after challenge. Hematoxylin and eosin-stained cross-sections of limb vessels dorsal to the heparin infusion site are shown. Leukocytes adhere to vessel walls (green arrows) in untreated animals (A) and transmigrate through the endothelial barrier (asterisk, higher magnification) (B). Corresponding sections of Icat-treated animals are shown in (C) and at higher magnification in (D). Scale bars represent 250 μm in (A) and (C) and 10 μm in (B) and (D). Representative histology of n = 7 rats in each group is shown.

(E) Heparin was injected into the aorta of rats and the mean arterial blood pressure was measured in the left carotid artery in untreated (red line) or Icat-pretreated (blue line) rats. The times of Icat and heparin administration are indicated by white and black triangles, respectively. Means ± SD, n = 7 rats.

(F) Arterial blood pressure during IgE/Ag-induced systemic anaphylaxis in WT (red), $F12^{-/-}$ (black), and $Bdkrb2^{-/-}$ (green) mice or WT mice pretreated with PCK (gray) or Icat (blue). Infusion of Icat or PCK and DNP-HSA is symbolized by white and black triangles, respectively. Means ± SD of 10 mice per group are displayed.

(G) Immunoblot analyses of plasma with antibodies against HK (nonprocessed single-chain HK; top), the BK sequence in HK (middle), and FXI (bottom) in plasma of IgE/Ag-treated (lane 1), IgE/buffer-challenged (lane 2), and buffer-treated (lane 3) mice (G).

Mast Cell-Heparin Increases Microvascular Permeability in a BK-Dependent Manner in Mice

A hallmark of BK-mediated effects is increased vascular permeability (Han et al., 2002). To analyze contact system-mediated leakage in genetically altered mice, we established an intravital confocal scanning microscopy technique to visualize microvascular fluid efflux.

anaphylactic mice (Figure 4G). The activated partial thromboplastin time (aPTT) is a commonly used diagnostic coagulation test to measure heparin plasma activity. IgE-Ag challenge largely prolonged the aPTT (>180 s versus 46 s in buffer-treated animals, n = 15 mice each), corresponding to a heparin plasma concentration of 4.7 ± 1.7 μg/ml, exceeding the threshold for activating FXII and PK, and BK formation (Figure 1A). Both addition of the heparin-antidote protamine and heparinase normalized the prolonged aPTT in plasma of anaphylactic mice, confirming that the aPTT prolongation is due to heparin activity. Cumulatively, the data show that heparin initiates BK formation via the FXII-stimulated contact system and that this pathway contributes to mast cell-driven hypotonic reactions in systemic anaphylaxis in vivo.
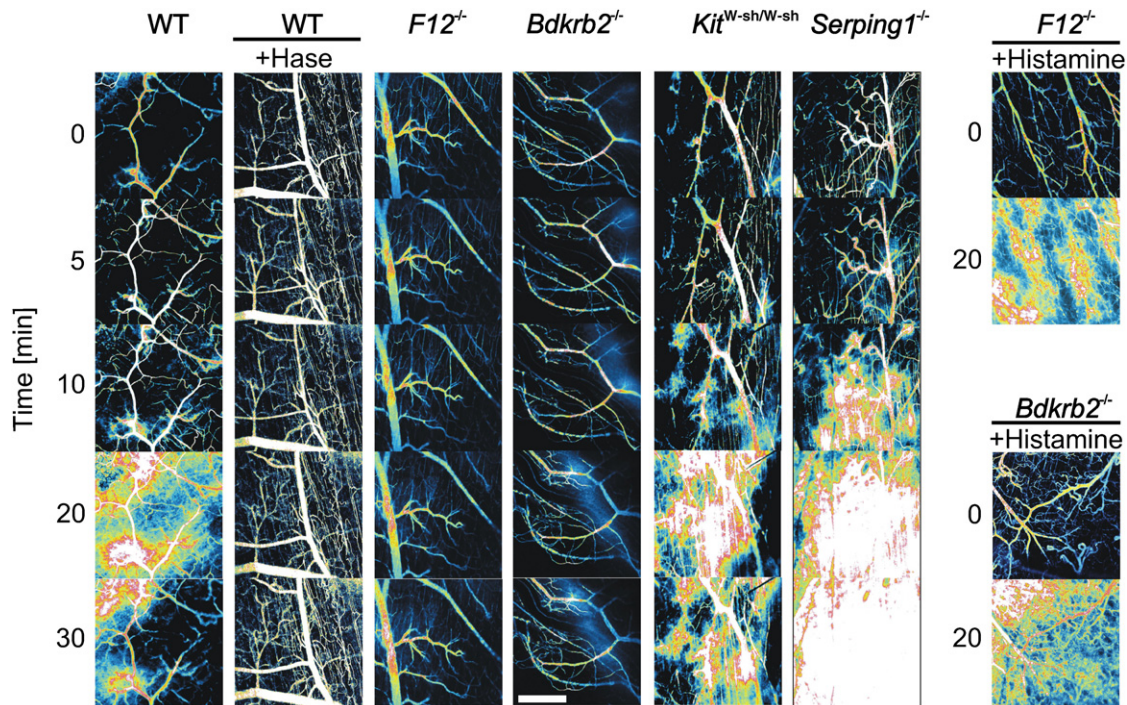
Macromolecular FITC-dextran was intravenously injected as a tracer for paracellular extravasated plasma proteins. A ventral skin window was incised, and skin was inverted and analyzed under the microscope. Fluorescence tracer showed blood flow in microvessels during the experiment. No basal tracer extravasation was detectable for 5 min prior to stimulation, indicating intact vascular barriers. Topical application of heparin (time point 0 min) provoked leakage from capillaries in WT mice. The initial leaky spots appeared within 10 min and leakage was maximal after 20 min (tracer fluorescence intensity 67.1-fold ± 15.2-fold of initial t = 0 min signal; Figure 5, column 1). Movie S1 is a 30 min time-lapse presentation of heparin-triggered FITC-dextran leakage from dermal vessels. To exclude that the increase in vascular permeability is mediated by a contaminant

Figure 5. MC-Heparin Increases Permeability of Skin Microvessels

Extravasation of FITC-dextran tracer from murine dorsal skin microvessels was recorded by intravital laser scanning fluorescence microscopy in real time. Heparin was topically applied to the skin of WT, $F12^{-/-}$, $Bdkrb2^{-/-}$, $Kit^{W-sh/W-sh}$, and $Serping1^{-/-}$ mice (columns 1, 3–6). For control, Hase-treated heparin was applied to the skin of WT animals (column 2) and histamine to $F12^{-/-}$ and $Bdkrb2^{-/-}$ mice (column 7). Laser scanning images were taken at 5, 10, 20, and 30 min after stimulation at time point 0 min and are shown in false colors. White represents the highest and black the lowest tracer intensity, respectively. The scale bar represents 500 μm. Confocal images were recorded with a 10× objective and processed with software EZ-C1, version 2.10 for Nikon. A typical experiment of a series of n = 10 mice per genotype is shown. Movies S1–S4 show time-lapse recordings of heparin-triggered leakage in mice.

rather than by heparin, we digested the polysaccharide with Hase. Hase treatment abolished the activity of the polysaccharide to increase vascular leakage in WT mice (1.6- ± 0.9-fold at 20 min; Figure 5, column 2). To exclude a contribution of released endogenous heparin, we tested mast cell-deficient mice ($Kit^{W-sh/W-sh}$). Exogenous heparin triggered leakage in mast cell-deficient mice (64.2- ± 4.2-fold; Figure 5, column 5) to a similar extent as observed in WT animals. Because FXII-independent mechanisms for PK activation exist (Schmaier, 2008), we analyzed heparin-triggered leakage in $F12^{-/-}$ mice that are defective in contact system-driven BK formation (Pauer et al., 2004). Intravital microscopy showed that $F12^{-/-}$ animals were resistant to MC-heparin-stimulated alterations in vascular permeability (1.6- ± 0.7-fold, 20 min; Figure 5, column 3; Movie S2). To confirm that heparin induces vascular leakage by releasing BK, we employed $Bdkrb2^{-/-}$ mice that are protected from BK-driven edema (Han et al., 2002). $Bdkrb2^{-/-}$ animals were mostly resistant to heparin-induced increase in permeability (1.7- ± 1.4-fold, 20 min; Figure 5, column 4, Movie S3). Histamine triggered leakage in FXII- and B2R-deficient mice (63.3- ± 8.7-fold and 65.8- ± 11.8-fold; Figure 5, column 7), indicating that these animals are susceptible to plasma contact system-independent edema formation.

Hereditary angioedema is characterized by recurrent swelling attacks. The underlying disease mechanism is a deficiency in functional C1INH (Zuraw, 2008). In patients with inherited C1INH deficiency, poorly defined stimuli trigger contact system-driven excessive BK formation that increases vascular leakage (Davis, 2008). To generate an animal model for HAE, we inactivated the gene encoding C1INH ($Serping1$) in mice, as described in the Supplemental Experimental Procedures. Because mast cells contribute to allergic angioedema (Green-hawt and Akin, 2007), we investigated heparin as a potential trigger for vascular leakage in C1INH-deficient ($Serping1^{-/-}$) mice. Intravital microscopy showed that heparin provoked excessive vascular leakage in $Serping1^{-/-}$ versus WT mice (compare Figure 5, column 6 versus 1; Movie S4 shows leakage in $Serping1^{-/-}$ mice). Leakage began earlier (first leaky spots at 4.5 min versus 10 min), was longer lasting (>50 min), and was greatly increased in $Serping1^{-/-}$ over WT mice (113- ± 17.2-fold versus 67.1- ± 15.2-fold, t = 20 min). Cumulatively, the data show that heparin directly triggers vascular leakage by FXII-dependent BK formation in vivo.

Mast Cell-Heparin Induces Contact System-Driven Edema in Mice

We analyzed the relative importance of heparin-driven BK formation for mast cell-mediated leakage in mice via the Miles edema model (Miles and Miles, 1952). Intradermally injected stimuli triggered leakage in dermal vessels that was visualized by Evans Blue tracer. Extravasated tracer was extracted from the tissue, quantified by fluorescence emission, and plotted relative to the
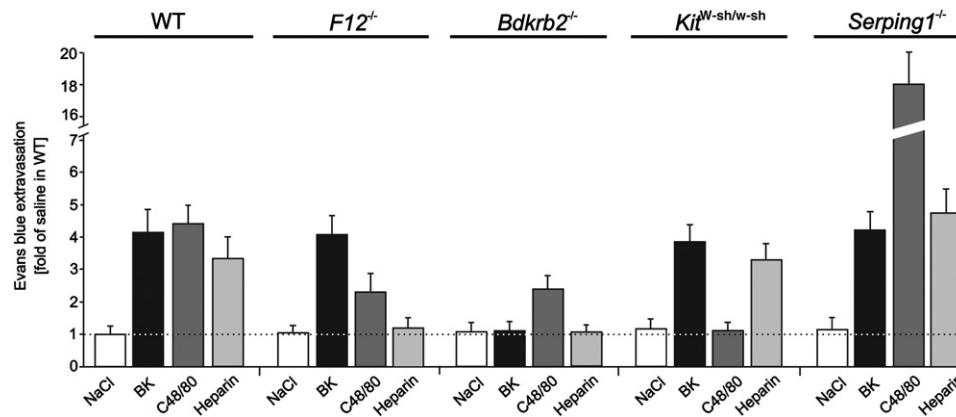
Figure 6. MC-Heparin Initiates Edema in a BK-Dependent Manner
Evans blue was infused intravenously as a tracer into WT, $F12^{-/-}$, $Bdkrb2^{-/-}$, $Kit^{W-sh/W-sh}$, and $Serping1^{-/-}$ mice. Skin edema was induced by intradermal injections with BK, C48/80, heparin, or saline. Leakage was analyzed after 30 min. Spots with extravasated tracer were excised entirely and dye was extracted and quantified. Tracer extravasation is plotted relative to leakage in WT mice stimulated with saline. Columns show means ± SD, n = 10 per group. Representative mouse hides are shown in Figure S3. Figure S1 shows effects of pharmacological inhibitors on heparin-induced edema formation.

saline-induced signal in WT mice (set to 1.0, Figure 6; Figure S3). Basal vascular permeability, assessed in saline-injected skin, was low (≤ 1.2) in all mice tested. BK-stimulated leakage was similar in WT and $F12^{-/-}$ mice (4.2- ± 0.9-fold versus 4.1- ± 0.7-fold) whereas $Bdkrb2^{-/-}$ animals were resistant to BK challenge (1.2- ± 0.5-fold). Heparin injection induced edema in WT animals (3.3- ± 0.8-fold), whereas $F12^{-/-}$ and $Bdkrb2^{-/-}$ mice were almost completely resistant to heparin-induced leakage and edema was not significantly different from saline controls (1.3- ± 0.4-fold versus 1.1- ± 0.3-fold and 1.1- ± 0.3-fold versus 1.1- ± 0.2-fold, p > 0.05). Because congenital deficiency in FXII and B2R provides protection from edema, we tested pharmacological inhibition of BK formation and signaling for interference with heparin-driven leakage. Infusion of PCK or Icatibant prior to challenge largely reduced heparin-induced edema in WT mice as compared to untreated animals (1.7- ± 0.5-fold and 1.5- ± 0.5-fold versus 3.0- ± 0.6-fold; Figures S1B and S1C). Tracer measurements are consistent with the intravital microscopy data (Figure 5). Cumulatively, the data show that heparin is an activator of the contact system and that the polysaccharide initiates BK-driven edema in vivo. To determine the importance of heparin-initiated BK formation for activated MC-driven skin edema, we challenged animals with compound 48/80 (C48/80), a degranulator of mast cells and eosinophils (Sher and Wadee, 1981). Mast cell-deficient mice were largely resistant to C48/80 stimulation and leakage was low as in saline controls (1.1- ± 0.4-fold versus 1.2- ± 0.3-fold). In $F12^{-/-}$ animals, C48/80-stimulated leakage was reduced to 2.3- ± 0.6-fold, i.e., 52% of WT mice permeability (4.4- ± 0.6-fold). $Bdkrb2^{-/-}$ mice confirmed that FXII-triggered BK formation, rather than other FXII-initiated pathways, is important for MC-induced edema. In these animals, C48/80-provoked leakage was reduced to 55% (2.4- ± 0.5-fold) compared to WT mice. The data indicate that heparin-induced BK formation accounts for about half of the edema-forming capacity of activated mast cells. To test heparin for triggering edema in HAE, we employed our $Serping1^{-/-}$ mice. Saline-induced leakage was 1.2- ± 0.4-fold of WT mice, indicating that basal vascular permeability in $Serping1^{-/-}$ mice was not

significantly different from that of WT animals (p > 0.05). In contrast, application of heparin and C48/80 triggered excessive edema that exceeded the leakage observed in WT mice by >45% and >60% (4.8- ± 0.7-fold and 18.1- ± 2.0-fold), respectively. However, BK-driven edema was not significantly different between $Serping1^{-/-}$ mice and WT animals (4.3- ± 0.6-fold; p > 0.05). Taken together, the data show that both heparin and activated mast cells trigger edema by generating BK.

Allergen-Stimulated Mast Cells Induce BK-Mediated Edema

To further evaluate the role of the plasma contact system for mast cell-mediated skin edema under pathophysiological conditions, we set up a model for passive cutaneous anaphylaxis, an IgE-dependent hypersensitivity reaction (Siebenhaar et al., 2008). Histamine was applied as positive control and induced comparable leakage in the skin of WT, $F12^{-/-}$, $Bdkrb2^{-/-}$, $Kit^{W-sh/W-sh}$, and $Serping1^{-/-}$ mice (4.4- ± 0.5-fold to 4.8- ± 1.0-fold; Figure 7; Figure S4). In contrast, injected Hase-treated heparin failed to increase leakage over saline (1.1- ± 0.4-fold to 1.3- ± 0.4-fold). We topically sensitized mice by intradermal anti-DNP IgE injection, challenged them 20 hr later with intravenous injection of DNP-HAS, and analyzed extravasated tracer after 30 min (Figure 7; Figure S4). Allergen exposure increased leakage in WT animals to 26.6- ± 5.0-fold (Figures 7A and 7F), whereas $Kit^{W-sh/W-sh}$ mice were resistant to IgE-Ag-mediated leakage (1.2- ± 0.4-fold), supporting the specificity of the stimulus for MC-driven edema. Under the same conditions, edema was reduced to 14.6- ± 5.2-fold and 13.6- ± 6.6-fold in $F12^{-/-}$ and $Bdkrb2^{-/-}$ mice, respectively. The cutaneous anaphylaxis model is consistent with data from C48/80-stimulated mast cells and supports a critical role of BK for sensitized mast cell-driven leakage.

Allergens Exacerbate Edema in C1INH-Deficient Mice and in HAE Patients

To analyze allergen-stimulated mast cells for initiating edema in HAE, we challenged $Serping1^{-/-}$ mice in our skin anaphylaxis
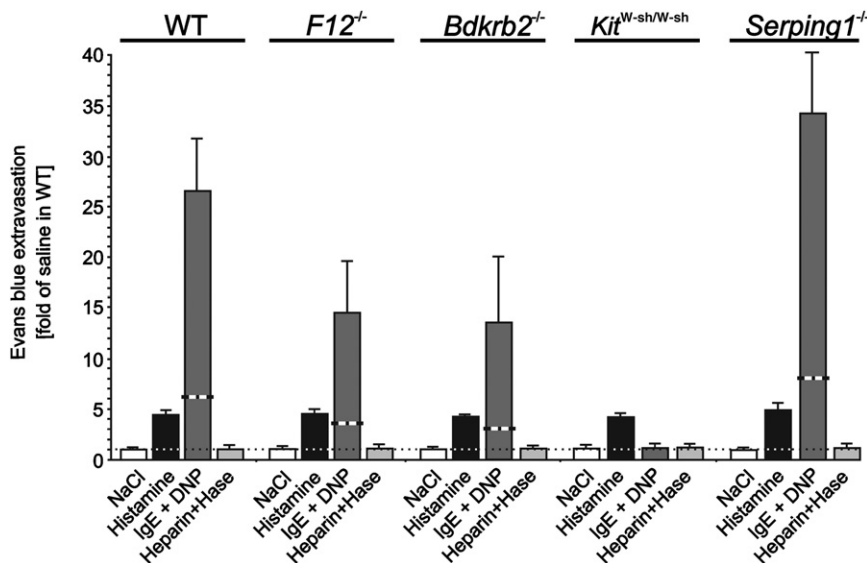
Figure 7. BK Contributes to Edema in a Mouse Model of Passive Cutaneous Anaphylaxis

Mice were intradermally injected with IgE or saline and challenged 20 hr later by intravenous injection of DNP-HSA and Evans blue tracer. Tracer was extracted after 30 min from the entire skin edema site from WT, $F12^{-/-}$, $Bdkrb2^{-/-}$, $Kit^{W-sh/W-sh}$, and $Serping1^{-/-}$ mice. Tracer fluorescence was quantified and signal is plotted relative to saline-induced extravasated Evans blue in WT mice (dashed lines represent tracer intensity in a circle of 1.2 cm diameter within the edema). Means ± SD, n = 10 per group are shown. Corresponding representative mouse hides are shown in Figure S4.

model. IgE-Ag-activated mast cells provoked excessive edema in $Serping1^{-/-}$ animals (34.1- ± 6.1-fold; p > 0.05 versus WT; Figure 7; Figure S4). The mouse HAE model identifies allergen-sensitized mast cells as an initiator of pathological edema formation, so we further tested the importance of this concept for the human disease state. In a survey, 38 well-characterized HAE patients from Switzerland were asked to report potential triggers of edema attacks. In 11 out of 38 HAE patients, the onset of swelling was associated with allergic reactions to food. In three individuals, edema formation was triggered by hyperimmune responses to insect toxins, and in a single patient, swelling episodes were induced by drug hypersensitivity.

In summary, the data identify MC-heparin as an in vivo initiator of the proinflammatory contact activation system and demonstrate that heparin-driven BK formation is an important component of MC-evoked vascular leakage. These findings suggest that heparin release may have broad clinical relevance in a variety of edema diseases associated with abnormal mast cell activation such as angioedema, urticaria, or allergy.

DISCUSSION

For more than 75 years, heparin has been widely used as an anticoagulant drug (Jorpes, 1935). The polysaccharide prevents the formation and extension of blood clots in the circulatory system. In rare occasions, heparin infusion leads to potentially life threatening side-effects. Within minutes, adverse reactions including drop in blood pressure, nausea, tachycardia, and edema in various tissues including the skin and respiratory tract have been reported (MacLaughlin et al., 2002). These symptoms are distinct from those of heparin-induced thrombocytopenia (HIT; a prothrombotic immune-mediated disorder) and reminiscent of an acute allergic reaction (Warkentin and Greinacher, 2009). However, the lack of urticaria and pruritis argue against a contribution of mast cell- or basophil-derived histamine or serotonin in the pathology of these adverse effects (Schwartz, 2008). Our studies offer a rationale for immediate adverse heparin effects:

we show that heparin initiates BK generation by activating the FXII-driven contact system in vivo and that this pathway contributes to MC-driven inflammation, hypotension, and edema. Supporting the importance of BK for adverse heparin effects in patients, affected individuals have a severe cough (MacLaughlin et al., 2002). Elevated plasma concentrations of BK directly trigger coughing, and cough is a classical side effect during therapy with angiotensin converting enzyme (ACE) inhibitors. ACE inhibitors block the major BK-degrading enzyme and increase BK plasma concentrations (Leeb-Lundberg et al., 2005). Vice versa, risk and severity of MC-triggered edema is increased in patients with low ACE activity (Summers et al., 2008), whereas incidence of MC histamine-triggered urticaria is independent of ACE activity (Akcali et al., 2008).

Since November 2007, lethal acute hypersensitivity reactions in patients receiving commercial heparin largely increased in number (http://www.fda.gov/cder/drug/infopage/heparin/adverse_events.htm). A contaminant was identified in suspect preparations of heparin that was characterized as a nonnatural occurring oversulfated chondroitin sulfate (OSCS) (Guerrini et al., 2008). OSCS-contaminated heparin has a greatly increased potency for activating FXII and triggering kallikrein-mediated BK formation in human plasma and in a model of experimental hypotonic shock in vivo (Kishimoto et al., 2008). Infusion of the contact system activator dextran sulfate in pigs induced transient systemic hypotension, and Icatibant blocked the drop in blood pressure (Siebeck et al., 1994). These data are consistent with heparin-triggered BK formation and hypotension in rats and mice and support a role of BK in immediate adverse reactions triggered by negatively charged polysaccharides. BK-forming activity seems to be dependent on negative charge density of the polysaccharide rather than on a defined structure. In a reconstituted system, the potency to contact-activate FXII decreased from dextran sulfate and OSCS (4 sulfate residues per disaccharide in average; Guerrini et al., 2008) to heparin (having 2.7 sulfate residues per disaccharide), whereas heparan sulfate (1 sulfate residue; Capila and Linhardt, 2002) failed to function as contact system activator (Hojima et al., 1984). Besides heparin, mast cell granules contain other negatively charged polysaccharides, e.g., chondroitin sulfate E (Stevens et al., 1988). Chondroitin sulfate E is a potent activator of FXII in plasma (Hojima et al., 1984), is

resistant to Hase digestion (Linker and Hovingh, 1972), and may account for the minor FXIIa-generating activity in Hase-treated MC-released material. The potency to activate the plasma contact system greatly varies among diverse heparin preparations (Brunnée et al., 1997; Noga et al., 1999), reflecting differences in purification procedures, sources of the polysaccharides, and experimental settings. Some purification procedures of clinically used heparins fragment the polysaccharide backbone and modify its structure (Fareed et al., 1989).

Heparin appears to have a dual function in FXII activation. The polysaccharide provides a negatively charged surface that binds to and induces FXII autoactivation. Concomitantly, antithrombin III (ATIII), an irreversible inhibitor of FXIIa, binds to a specific pentasaccharide sequence with a unique 3-O-sulfated glucosamine unit within the heparin backbone (Capila and Linhardt, 2002). Theoretical models predict a threshold of complete FXII activation or inactivation that is determined by the kinetic balance between the catalytic rate of autoactivation and the rate of FXIIa inhibition (Beltrami and Jesty, 1995). Indeed, heparin-triggered FXII activation results in a bell-shaped dose response, which is also observed in OSCS-triggered contact system activation (Kishimoto et al., 2008).

Ablation of N-deacetylase N-sulfotransferase-2 (NDST-2) in mice revealed a crucial function of the enzyme for heparin biosynthesis. NDST-2-null ($Ndst2^{-/-}$) mice are deficient in sulfated heparin and have fewer mast cells (Forsberg et al., 1999; Humphries et al., 1999). $Ndst2^{-/-}$ mast cells have an altered morphology and less secretory granules that do not contain heparin and show a general deficiency in releasable mediators including various mast cell proteases. IgE-mediated secretion of histamine is defective in NDST-2-null mice, suggesting that heparin regulates MC granule content and function (Humphries et al., 1999). Because of combined deficiency of MC-heparin with other mediators such as histamine in $Ndst2^{-/-}$ mast cells, these animals do not allow researchers to specifically address the relative importance of MC-heparin-driven BK formation for vascular permeability. IgE-Ag-stimulated leakage in MC-deficient $Kit^{W-sh/W-sh}$ mice reconstituted with $Ndst2^{-/-}$ mast cells had high intra-animal variability. Therefore, we compared IgE-Ag-stimulated edema in $Kit^{W-sh/W-sh}$ mice versus animals with normal MC function versus mice with selective defects in BK formation or signaling.

During anaphylactic shocks, the aPTT is markedly prolonged in patients (Lombardini et al., 2009; Mazzi et al., 1994). Consistently, plasma of IgE-Ag-challenged mice is unclottable because of a systemic heparin concentration of >4 μg/ml, which is sufficient to initiate BK formation. Initially, small amounts of locally secreted heparin may generate BK activity on the MC surface. Mast cells express B2R (Dlamini and Bhoola, 2005) and BK stimulation induces MC degranulation (Ishizaka et al., 1985). Because of this amplification loop, an initial BK activity might be multiplied by liberated MC-heparin that triggers the contact system. FXIIa may initiate several protease cascades in plasma, such as the kallikrein-kinin system, the intrinsic pathway of coagulation, and the complement and fibrinolytic systems (Müller and Renné, 2008). We recently showed that FXIIa-initiated fibrin formation is essential for arterial thrombus formation (Renné et al., 2005a) and ischemia reperfusion injury in experimental stroke models (Kleinschnitz et al., 2006). Procoagulant FXII

activity is initiated by a platelet-secreted inorganic polymer, polyphosphate. Polyphosphate-activated FXII activates its substrates FXI and PK, driving the intrinsic pathway of coagulation and the kallikrein-kinin system, respectively (Müller et al., 2009). In contrast, heparin specifically triggers FXII-mediated BK formation. Under these conditions, no proteolytic activation of FXI is detectable (Figure 2). Similarly, both the nonnatural polysaccharide dextran sulfate (Johne et al., 2006) and misfolded protein aggregates initiate BK formation in a FXII-dependent manner (Maas et al., 2008). However, these agents do not induce clotting activity. Because FXI and PK have similar plasma concentrations and highly homologous structures and are both bound via HK to cell surfaces (Renné et al., 2005b), mechanisms for cell type-specific activation of the kallikrein-kinin system and/ or the intrinsic clotting pathway may exist that involve distinct FXII-activation mechanisms, different forms of FXIIa (Schmaier, 2008), effects of the activators on downstream inhibitors (Kishimoto et al., 2008), and other yet unknown regulators.

Supporting the concept of selective activation of FXII-driven pathways, HAE patients suffer from recurrent BK-mediated swelling but edema attacks are not associated with any increased prothrombotic risk (Nzeako et al., 2001). Besides the classical HAE types I and II that are due to a deficiency in or a dysfunctional C1INH, respectively, a third variant (HAE type III) with a normal C1INH plasma concentration and function exists. HAE type III is due to a gain-of-function mutation in FXII that increases the enzymatic activity of the protease (Cichon et al., 2006). Similar to our study involving HAE type I and II patients (Figure 7), edema in HAE type III patients is triggered by allergen exposure (Bork et al., 2009), supporting a role of heparin for initiating BK-mediated edema in humans. HAE patients (Zuraw, 2008) and our $Serping1^{-/-}$ mice (Figure 6) have normal vascular permeability under nonstimulated conditions. In contrast, previously generated C1INH-deficient mice have increased basal vascular permeability (Han et al., 2002). The reason for the different phenotypes of the two mouse strains might be due to different technologies used to inactivate the $Serping1$ gene coding for C1INH. We used random mutagenesis, which results in early and complete termination of C1INH protein synthesis, whereas Han and coworkers used a random insertional mutagenesis technique (gene trap). The latter technique targets the $Serping1$ gene through a retroviral vector that codes for galactosidase and a selection marker. The trapping vector is inserted into a splice-acceptor site in intron 6 (210 bp 5′ of exon 7). This approach does not abolish $Serping1$ gene expression but results in transcription of a C-terminal shortened C1INH variant composed of exons 1–6 (amino acids 1–347 of 504 in the mature protein) fused to trap-vector coded β-galactosidase (Ullrich and Schuh, 2009). The biological relevance, i.e., stability, localization, and interactions, with other proteins of this truncated C1INH variant is not clear. Although the mutant lacks the active site (encoded by exon 8), it includes the signal peptide and could be secreted into the plasma or may modulate intracellular pathways that alter basal permeability.

Cumulatively, this study identifies mast cell-heparin as an in vivo activator of the FXII-driven proinflammatory plasma contact system. Heparin-initiated BK formation substantially contributes to swelling, anaphylactic, and inflammatory diseases associated with aberrant mast cell activity.

EXPERIMENTAL PROCEDURES

Vascular Permeability Assays

To analyze vascular leakage in real time with intravital laser scanning microscopy, we adapted a method originally described for leukocyte infiltration into vascular lesions (Eriksson et al., 2001). Mice were anesthetized by intraperitoneal injection of Avertin (2,2,2-tribromoethanol and 2-methyl-2-butanol). Then, 3 ng/g bw FITC-dextran (150 kDa; Sigma-Aldrich) was injected into the retroorbital plexus as tracer. A ventral skin window was excised and the skin was fixed plain. Extravasation of tracer from microvessels (35–60 μm diameter) was visualized with a Nikon Eclipse E600 microscope equipped with a C1 laser scanning head and a 10× objective. Leakage was assessed for 5 min before and 30 min after topical application of heparin (20 μl of a 1 mg/ml solution). Tissue scans were performed at 30 s intervals. Images were analyzed by EZ-C1 2.10 software (Nikon) and the intensity of extravasated tracer was quantified with ImageJ 1.34 NIH software. Dorsal skin edema was induced by intradermal injections of 50 μl heparin (1 mg/ml), C48/80 (50 μg/ml), saline, histamine (100 μM), BK (100 μM), or Hase-treated heparin (10 mg/ml) (Miles and Miles, 1952). Some WT mice were pretreated with PCK (8 μg/g bw), Icatibant (100 μg/kg bw), or saline 5 min prior to heparin injection. After 30 min, the mice were sacrificed and the skins were removed and photographed. The edema site was excised with a circular template of 1.2 cm diameter. For edema, with diameter >1.2 cm the entire blue area was excised by hand. Extravasated Evans blue tracer was quantified as described (Donelan et al., 2006).

Anaphylaxis Models

To induce passive cutaneous anaphylaxis, mice were sensitized by intradermal injection of anti-DNP IgE antibody (1.5 μg/ml) or 50 μl sterile 0.9% NaCl (control) in the dorsal skin. After 20 hr, we challenged mice by retro-orbital injection of dinitrophenyl-human serum albumin (7.5 mg/kg bw, DNP-HSA) together with 10 μl/g bw of 0.25% Evans Blue as described (Siebenhaar et al., 2008) with minor modifications. Tracer extravasation was measured as above. For the passive systemic anaphylaxis model, we injected mice intravenously with anti-DNP IgE (1.25 μg/g bw), challenged them 24 hr later by intravenous injection of 1 mg DNP-HSA, and recorded arterial blood pressure by fluid-filled catheter connected to a pressure transducer (APT 300, Harvard Apparatus).

Heparin-Induced BK Generation in Rats

Male rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p., Nembutal, Ceva). Catheters were placed into the carotid artery and vena cava and into the jugular artery and vein. The rats were placed in a supine position on a heated operating pad. Mean systolic arterial blood pressure was measured in the left carotid artery with an arterial catheter connected to a Statham pressure transducer (type P 23 I, Gould) and recorder (LS 64, Linseis). We applied a bolus of 10 mg/kg bw heparin into the aorta or femoral artery to trigger hypotension or inflammation, respectively, and continuously monitored mean arterial blood pressure and vital signs of the animals during challenge. For some experiments, rats were treated with Icatibant (100 μg/kg bw) prior to heparin challenge. Plasma was collected from the vena cava for BK determination and immediately supplemented with protease inhibitors. Frozen cross-sections of the A. femoralis and V. femoralis 2 mm caudal to the knee joint were stained with haematoxylin-eosin 30 min after heparin application. All experiments and animal care were approved by the local Animal Care and Use committee.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four movies and can be found with this article online at doi:10.1016/j.immuni.2011.02.008.

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