Plasma contact system activation drives anaphylaxis in severe mast cell–mediated allergic reactions

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Background: Anaphylaxis is an acute, potentially lethal, multisystem syndrome resulting from the sudden release of mast cell–derived mediators into the circulation.

Objectives and Methods: We report here that a plasma protease cascade, the factor XII–driven contact system, critically contributes to the pathogenesis of anaphylaxis in both murine models and human subjects.

Results: Deficiency in or pharmacologic inhibition of factor XII, plasma kallikrein, high-molecular-weight kininogen, or the bradykinin B2 receptor, but not the B1 receptor, largely attenuated allergen/IgE-mediated mast cell hyperresponsiveness in mice. Reconstitutions of factor XII null mice with human factor XII restored susceptibility for allergen/IgE-mediated hypotension. Activated mast cells systemically released heparin, which provided a negatively charged surface for factor XII autoactivation. Activated factor XII generates plasma kallikrein, which proteolyzes kininogen, leading to the liberation of bradykinin. We evaluated the contact system in patients with anaphylaxis. In all 10 plasma samples immunoblotting revealed activation of factor XII, plasma kallikrein, and kininogen during the acute phase of anaphylaxis but not at basal conditions or in healthy control subjects. The severity of anaphylaxis was associated with mast cell degranulation, increased plasma heparin levels, the intensity of contact system activation, and bradykinin formation.

Conclusions: In summary, the data collectively show a role of the contact system in patients with anaphylaxis and support the hypothesis that targeting bradykinin generation and signaling provides a novel and alternative treatment strategy for anaphylactic attacks. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Anaphylaxis, mast cell, bradykinin, mouse models, tryptase, contact system*

Anaphylaxis is a severe and often unanticipated multisystem syndrome of rapid onset and potentially fatal outcome. It most often represents an immunologic response to certain allergens, resulting in sudden systemic degranulation of mast cells and basophils.[1-3] Common triggers of anaphylaxis include food, medications, insect venoms, and other allergens cumulatively affecting as much as 1% to 15% of the population, with an increasing prevalence.[4] Anaphylaxis typically manifests with a broad range of symptoms, such as hypotension, vascular leakage, cardiac arrhythmias, bronchial constriction, and gastrointestinal and skin manifestations. Anaphylactic shock represents the most dramatic and potentially catastrophic manifestation of immediate hypersensitivity[5] and might account for more than 500 deaths annually in the United States.[6] Although the mechanisms have not been completely elucidated, systemic hypotension and circulatory shock are believed to result from peripheral vasodilatation, enhanced vascular permeability, plasma leakage, and intravascular volume depletion rather than a direct effect on the myocardium.[7] Despite recent advances in our understanding of the mechanisms and mediators involved in anaphylaxis, its typically acute and unforeseen presentation often hampers treatment.

In most cases anaphylaxis is initiated by an antigen (allergen) interacting with allergen-specific IgE bound to FcεRI on mast cells, basophils, or both; however, other immunologic and

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Abbreviations used
ACE: Angiotensin-converting enzyme
Anti-Xa: Anti–factor Xa activity
aPTT: Activated partial thromboplastin time
Bdkrb1: B1R coding gene
Bdkrb2: B2R coding gene
B1R: Bradykinin B1 receptor
B2R: Bradykinin B2 receptor
C1INH: C1 esterase inhibitor
DNP-HSA: Dinitrophenyl–human serum albumin
FXI: Factor XI
FXII: Factor XII
FXIIa: Activated factor XII
HAE: Hereditary angioedema
HK: High-molecular-weight kininogen
IQR: Interquartile range
Klkb1: Plasma kallikrein coding gene
Kng1: High-molecular-weight kininogen coding murine gene
MABP: Mean arterial blood pressure
NO: Nitric oxide
PCK: H-D-Pro-Phe-Arg-chloromethyl ketone
PK: Plasma kallikrein
PRCP: Prolylcarboxypeptidase
WT: Wild-type

nonimmunologic mechanisms exist.[5] The allergen/IgE complex initiates intracellular signaling that results both in release of preformed but also *de novo* synthesis of mediators, enzymes, and cytokines, including leukotrienes, histamine, the proteases tryptase and chymase, carboxypeptidase A, and proteoglycans. These highly sulfated polysaccharides, with heparin as the major component on a weight basis, are abundant in mast cell secretory granules and released on degranulation. *In vivo* heparin is exclusively synthesized in mast cells and contributes to the morphology and storage capacity of their secretory granules.[2] Purified and activated mast cell–released heparin provides the negatively charged surface for binding of the plasma protease factor XII (FXII; Hageman factor). Binding to a surface induces a conformational change in zymogen FXII, a process known as autoactivation (contact activation). Activated FXII (FXIIa) activates plasma kallikrein (PK) zymogen to the active protease, which in turn proteolytically generates the peptide hormone bradykinin from its precursor, high-molecular-weight kininogen (HK).[8] Bradykinin acts on G protein–coupled bradykinin B2 receptors (B2Rs) to increase vascular permeability.[9] Fig 6 shows a schematic overview of the FXIIa-driven contact system reaction cascade. Mast cell heparin triggers FXIIa-mediated bradykinin formation in human plasma *in vitro*[10,11] and increases vascular permeability in genetic mouse models of anaphylaxis.[12] Experimentally induced reactive nasal airway allergy locally increases bradykinin levels,[13-15] and bradykinin levels are increased in patients with allergic rhinitis.[16] Together the data suggest a role for bradykinin in local allergic reactions; however, clinical evidence for systemic activation of the contact system and ways to incorporate targeting bradykinin in the therapeutic management of anaphylaxis have remained underdeveloped.[17]

Here we analyze a potential role of the plasma contact system for anaphylaxis *in vivo* using a combination of contact system protein–deficient and humanized animal models and diseased patient plasma. The study shows a role of contact system–produced bradykinin in severe hypersensitivity reactions and suggests targeting bradykinin generation and its downstream signaling as a promising strategy for interference with anaphylaxis and possibly other immunologic disorders.

METHODS

Patients

We included all adult (≥18 years old) patients with anaphylaxis treated at the Department of Internal Medicine, Allergy Section of the University Hospital Vall d'Hebron, Barcelona, Spain, between June and August 2011. Only patients who fulfilled the definition of anaphylaxis with an allergy work-up that confirmed the diagnosis and ruled out other disease, had at least a serum and plasma sample taken during the episode and at baseline, and signed an informed consent form were included. We defined anaphylaxis according to the 2006 National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network criteria.[18] Anaphylaxis severity was classified according to the grading system based on clinical symptoms, as previously described,[19] according to the Brown classification (Table I).[20] The moderate group was subdivided into moderate A (grade 1, with gastrointestinal symptoms) and moderate B (grade 2, with respiratory symptoms). Grade 3 classifies severe anaphylaxis with hypotension.

Patients were followed up at the outpatient clinic of Vall d'Hebron University Hospital Allergy Section, where an allergy work-up was performed as needed (skin prick tests, specific IgE measurements, and/or challenge tests) and the diagnosis of anaphylaxis was confirmed by an allergist. Conditions that mimic anaphylaxis (eg, anxiety disorders, vocal cord dysfunction, or mastocytosis) were ruled out in all patients. We also obtained control plasma and serum samples from 10 age- and sex-matched patients, 4 atopic (3 with rhinitis and asthma caused by house dust mites and 1 with a history of urticaria caused by food allergy to peach) and 6 nonatopic patients, seen at the outpatient clinic of the same hospital between June and August 2011 for follow-up and treatment. All control subjects were asymptomatic, with no reported signs of allergy at the time of sampling. The ethics committee of Vall d'Hebron University Hospital approved the study (PR 53/2009), and all samples were collected with signed informed consent of the participants.

Animals

All animal care and experimental procedures complied with the Principles of Laboratory and Animal Care established by the National Society for Medical Research and were approved by the Bezirksregierung of Unterfranken or Stockholm's Norra Djurförsöksetiska Nämnd. *F12*$^{-/-}$, *FXI*$^{-/-}$, *Bdkrb1*$^{-/-}$, *Kng1*$^{-/-}$, and *Bdkrb2*$^{-/-}$ mice were backcrossed for more than 10 generations to the C57Bl/6 background, as previously described.[21,22] All progeny were genotyped by using PCR. All studies were performed on male mice 6 to 8 weeks of age. Age- and sex-matched wild-type (WT) control mice were purchased from Charles River (Wiga, Sulzfeld, Germany).

Generation of *Klkb1*$^{-/-}$ mice, genotyping, and expression analysis

The *Klkb1*$^{-/-}$ mice were generated by using a homologous recombination-based targeting strategy that replaces exon 1 (bp 1549 to 1567 in the murine PK gene of 2573 bp) with a Neo cassette. Successfully targeted C57Bl/6 embryonic stem cells were identified by means of Southern blotting of *Eco*RV/*Spe*I-digested isolated DNA from embryonic stem cells by using a probe with labeled DNA directed just outside the construct arm. We used genomic DNA from tail samples for PCR genotyping under the following conditions: denaturation at 94°C for 15 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 40 seconds. The PCR was run in 30 cycles with the following 3 primers for genotyping of WT-specific product: 5′-CCAATGTGACTCGTTTCCTGACTTG-3′, 5′-GATCCTAGTTGGGGAGCCATCTGTG-3′, and 5′-GGGTGGGATTAG ATAAATGCCTGCTCT-3′, which amplify fragments of 567 and 365 bp

TABLE I. Clinical data of patients with anaphylaxis and times of sampling

Patient no.	Time*	Sex	Age (y)	Etiology	Clinical manifestations				Severity of anaphylaxis	ACE inhibitor	Personal history of atopy	Comorbidities
					U	GI	R	CV				
1	30 min	F	40	ASA	+	−	+	−	2	No	No	No
	2 h											
	Basal											
2	1.5 h	F	28	Mango	+	−	+	−	2	No	BA, RC, FA	No
	6 h											
	Basal											
3	30 min	F	33	Infliximab	+	+	+	−	2	No	No	Ulcerative colitis
	2 h											
	Basal											
4	1.5 h	M	40	Lettuce and ASA	+	+	−	−	1	No	FA	No
	Basal											
5	3 h	F	20	Walnut	+	+	−	+	3	No	FA, RC	No
	Basal											
6	2 h	F	65	Orange	+	+	−	+	3	No	FA	
	Basal											
7	3 h	M	39	*Anisakis* species	+	+	−	−	1	No	No	No
	Basal											
8	30 min	M	41	ASA	+	−	+	−	2	No	No	No
	Basal											
9	2 h	F	42	Amoxicilin	+	−	+	+	3	No	No	No
	Basal											
10	10 h	M	79	Dipyrone	+	−	−	+	3	Yes	No	HBP
	Basal											

Severity of anaphylaxis is defined as follows: *1*, moderate A; *2*, moderate B; and *3*, severe.
ASA, Acetylsalicylic acid; *BA*, bronchial asthma; *CV*, cardiovascular symptoms; *F*, female; *FA*, food allergy; *GI*, gastrointestinal symptoms; *HBP*, hypertension; *M*, male; *R*, respiratory symptoms; *RC*, rhinoconjunctivitis; *U*, urticaria.
*Time when blood was drawn after the onset of anaphylaxis.

for targeted and endogenous *Klkb1*, respectively. Plasma samples of 0.2 μL per lane were separated under reducing conditions on 10% SDS-PAGE, electrotransferred to nitrocellulose membranes, and probed with anti-PK, anti-HK, and anti-FXII antibodies, as described below.

Anaphylaxis models

Mice were intravenously injected with anti-DNP IgE mAb (1.25 μg/g body weight; Sigma-Aldrich, Steinheim, Germany) and challenged 24 hours later by means of intravenous injection of 1 mg of dinitrophenyl–human serum albumin (DNP-HSA; Sigma-Aldrich) in 100 μL of 0.9% NaCl to induce systemic anaphylaxis. WT mice were intravenously treated with rHA-infestin-4 (kindly provided by Dr Marc Nolte, CSL Behring, Marburg, Germany); H-D-Pro-Phe-Arg-chloromethyl ketone (PCK); Fmoc-Ala-Pyr-CN (Bachem, Bubendorf, Switzerland); icatibant (HOE 140, Firazyr; donated by Shire, Berlin, Germany), DX-88 (Dyax, Boston, Mass); the anti-bradykinin antibody MBK3, which interferes with contact system–mediated HK cleavage[23]; R-715 (AcLys-[D-βNal[7],Ile[8]]desArg[9]-bradykinin; Tocris Bioscience, Bristol, United Kingdom; F. Raslan, 2010); or N5-(imino[methylamino]methyl)-L-ornithine citrate (Sigma-Aldrich) 10 minutes before DNP-HSA injection. Temocaprilat (Santa Cruz Biotechnology, Santa Cruz, Calif) was subcutaneously applied 2 hours before challenge. Human FXII zymogen (2 μg/g body weight; Haematologic Technologies, Essex Junction, Vt) was intravenously infused 10 minutes before allergen challenge. Arterial blood pressure in the carotid artery was recorded by using a fluid-filled catheter connected to a pressure transducer (APT 300; Harvard Apparatus, Holliston, Mass). Back skin temperature was recorded with a digital infrared thermometer (Kintrex IRT0421; Kintrex, Vienna, Va).

Analysis of contact system activation

Human or murine citrate-anticoagulated plasma was immediately frozen at −20°C and thawed by addition of SDS-PAGE sample buffer containing 8% (mass/volume) SDS. A volume of 0.25 μL of plasma per lane was separated by means of SDS-PAGE and analyzed by using Western blotting with antibodies to FXII, FXIIa (kindly donated by Dr David Pritchard, Axis Shield, Dundee, Scotland), PK (αPK2), HK (I108), and the bradykinin sequence in HK (MBK3) and a horseradish peroxidase–coupled secondary antibody, as previously described.[12,23,24] Detection was performed with a chemiluminescence technique (ECL Plus; Amersham Pharmacia Biotech, Little Chalfont, Bucks, United Kingdom). The intensity of the individual bands on x-ray films (XBA, Fotochemische Werke Berlin, Berlin, Germany) was quantified by means of densitometric scans with the National Institutes of Health ImageJ 1.37 software, as previously described (http://www.navbo.info/DensitometricAnalysys-NIHimage.pdf). Curve fitting was done with Prism 5.0 software (GraphPad Software, La Jolla, Calif). Bradykinin plasma concentrations were determined with the MARKIT-M-Bradykinin ELISA, according to the manufacturer's instructions (Dainippon Pharmaceutical, Osaka, Japan). Citrate plasma was incubated with dextran sulfate (500,000 Da, Sigma-Aldrich) for 20 minutes at 37°C to induce complete processing of plasma HK and supplemented with an inhibitor cocktail, including Pefabloc SC (Roth, Karlsruhe, Germany), phenylmethylsulfonyl fluoride (Calbiochem, Billerica, Mass), and the Serine Protease Inhibitor Cocktail Set I (Merck, Darmstadt, Germany) and SDS-PAGE sample buffer before mixing with untreated samples.

Coagulation assays

Mouse plasma collected into 3.2% sodium citrate was used for determination of the aPTT, according to existing protocols for aPTT determinations in human plasma, as described previously with minor modifications.[24] When indicated, plasma samples were pretreated with 0.5 U/mL heparinase for 30 minutes at 37°C or supplemented with 1 to 2 μg/mL protamine sulfate, which normalized prolonged aPTT. Anti–factor Xa activity (anti-Xa) was measured on a BCS XP 1.1 with the COAMATIC Heparin assay (Chromogenix, Milan, Italy).

Tryptase analysis

Serum tryptase levels were measured with the UniCAP-Tryptase fluoroimmunoassay (Thermo Fisher Scientific, Uppsala, Sweden), according to the manufacturer's protocol (http://www.phadia.com/en/Health-Care-Providers/Allergy/Products1/ImmunoCAp-Tryptase).

Data analysis

Data were collected and analyzed with Prism 5 software (GraphPad Software). All animal data are presented as the means of at least duplicate determinations and as means ± SDs, unless otherwise indicated in the text. In graphs error bars indicate ± 1 SE. All patient data are reported as medians and interquartile range (IQRs). Continuous variables were compared with a categorical variable by using the nonparametric Kruskall-Wallis and Mann-Whitney *U* tests. The Wilcoxon test was used for paired data. Correlations were analyzed by using the Spearman rank test. All statistical tests were 2-tailed, and a *P* value of .05 or less indicates statistical significance. Nonsignificant results represent a *P* value of greater than .05.

RESULTS

FXII null mice and mice with deletion of the B2R gene are protected from systemic hypotension during anaphylactic reactions

To characterize the functions of the plasma contact system during IgE-mediated immunologic responses, we evaluated WT and FXII null mice ($F12^{-/-}$) in a model of passive systemic anaphylaxis. $F12^{-/-}$ animals have previously been described and exhibit a defective ability to activate the FXII-driven contact pathway[25] but respond normally to injected bradykinin.[12] Mice were infused with anti-DNP–IgE and challenged 24 hours later with an intravenous injection of the corresponding antigen, DNP-HSA. Systemic mean arterial blood pressure (MABP) was measured at baseline, before DNP-HSA, and after antigen injection. We observed that both WT and $F12^{-/-}$ mice had stable MABPs, with little variation (102 ± 11 mm Hg) at baseline. WT mice responded to DNP-HSA injection with a rapid and transient decrease in MABP of 59 ± 24 mm Hg. In contrast, the allergen-induced hypotonic response was largely attenuated in $F12^{-/-}$ mice (23 ± 10 mm Hg; Fig 1, *A*). We targeted inactivated PK coding gene *(Klkb1)* expression to generate PK-deficient mice. Western blotting and aPTT (a measure of contact system–driven coagulation) clotting assays confirmed deficiency in plasma PK levels, whereas levels of other contact system proteins (FXII, HK, and factor XI [FXI]), as well as prothrombin time (a measure of tissue factor–driven coagulation), were in the normal range in these animals (see Fig E1 in this article's Online Repository at www.jacionline.org). $Klkb1^{-/-}$ animals were protected from an IgE/antigen-triggered decrease in MABP to a similar extent as $F12^{-/-}$ animals (30 ± 7 mm Hg), whereas mice deficient in the FXIIa substrate of the intrinsic coagulation pathway, FXI ($FXI^{-/-}$ mice, which shares high homology with PK[26]), were as susceptible to allergen-induced hypotension as WT control mice (63 ± 24 mm Hg, $P > .05$ vs WT mice).

We analyzed high-molecular-weight kininogen coding murine gene $(Kng1)^{-/-}$ mice, which are deficient in plasma HK,[21] and $Bdkrb2^{-/-}$ mice, which are deficient in B2R and thus resistant to bradykinin signaling,[23] in our model of passive systemic anaphylaxis. $Kng1^{-/-}$ and $Bdkrb2^{-/-}$ mice were protected from the IgE/antigen-activated decrease in MABP, and the degree of protection was similar to that observed in $F12^{-/-}$ and $Klkb1^{-/-}$ mice (26 ± 9 and 29 ± 10 mm Hg).

Des-Arg[9]-BK, the cleavage product of bradykinin, is the principal ligand for the kinin B1 receptor (B1R) and is generated by carboxypeptidases after removal of the C-terminal arginine residue from bradykinin.[9] In animal studies stimulation of B1R produces vasodilatation and a reduction in blood pressure.[27] IgE/antigen challenge induced a strong hypotonic reaction in mice deficient in the kinin B1 receptor coding gene $(Bdkrb1)^{-/-}$ that was similar to that seen in WT control animals ($Bdkrb1^{-/-}$ mice: 60 ± 22 mm Hg, $P > .05$ vs WT mice), indicating that bradykinin, but not its metabolite, is a mediator of mast cell immune-mediated hypotensive responses. To confirm that systemic anaphylaxis triggers FXII activation, resulting in sequential proteolytic activation of the FXII/PK/HK cascade and leading to bradykinin generation, we collected blood 10 minutes before and 20 minutes after DNP-HSA infusion and probed for FXII and PK zymogens, FXIIa, single-chain HK, and bradykinin using Western blotting. Compared with baseline conditions (preceding DNP-HSA infusion; Fig 1, *B*), we observed complete activation of FXII, PK, HK cleavage, and bradykinin liberation in WT, $FXI^{-/-}$, $Bdkrb2^{-/-}$, and $Bdkrb1^{-/-}$ mice. FXII and PK were also activated in $Kng1^{-/-}$ mice, which lack the PK substrate HK and thus are defective in contact system–driven bradykinin formation. Although FXII was activated in $Klkb1^{-/-}$ mice, HK cleavage was defective, and bradykinin was not liberated in these animals. In contrast, the bradykinin-forming protease cascade was not activated in $F12^{-/-}$ mice (Fig 1, *C*). Concomitant with FXIIa/PK-mediated HK cleavage and bradykinin liberation from its precursor molecule, plasma bradykinin levels rapidly increased in anaphylactic WT mice after antigen exposure up to peak plasma levels of 720 ± 220 ng/mL (Fig 1, *D*). Plasma bradykinin levels remained less than 75 ng/mL in challenged $F12^{-/-}$ mice. Endogenous heparin is exclusively found in mast cell granules, and circulating plasma heparin serves as a biomarker of mast cell activation.[28]

The aPTT is a commonly used diagnostic coagulation test that measures plasma heparin activity.[24] IgE/antigen challenge largely prolonged the aPTT from 25 ± 3 seconds before allergen stimulation to maximum levels (>150 seconds) after DNP-HSA infusion, corresponding to a heparin plasma concentration of greater than 5.0 μg/mL (Fig 1, *E*). Both addition of the heparin antidote protamine (10 μg/mL) and heparinase (1 U/mL), which degrades heparin, normalized the prolonged aPTT in plasma samples of anaphylactic WT mice. The aPTT is prolonged to 95 ± 10 seconds in unchallenged $F12^{-/-}$ mouse plasma[24] and increased to greater than 150 seconds after IgE/antigen challenge. Taken together, the gene-deficient mouse models indicate a role of bradykinin produced by the FXII/PK/HK reaction cascade that signals through B2R stimulation for IgE-triggered hypotension.

To confirm an effect of the contact system in mice with anaphylaxis, we measured cutaneous temperatures in IgE/antigen-challenged mice. We found a transient decrease in temperature, with the lowest values of 4.2°C ± 0.6°C, 4.2°C ± 0.3°C, and 4.0°C ± 0.6°C 30 minutes after infusion of DNP-HSA in IgE-sensitized WT, $FXI^{-/-}$, and $Bdkrb1^{-/-}$ mice, respectively. In contrast, FXII, PK, HK, and B2R deficiency significantly inhibited decreases in body temperature in sensitized mice (1.8°C ± 0.4°C, 1.6°C ± 0.5°C, 1.8°C ± 0.5°C, and 2.0°C ± 0.6°C, $P < .01$ vs WT mice; see Fig E2 in this article's Online Repository at www.jacionline.org). These combined data indicate that the contact system is active and contributes to adverse symptoms in systemic anaphylaxis in mice.

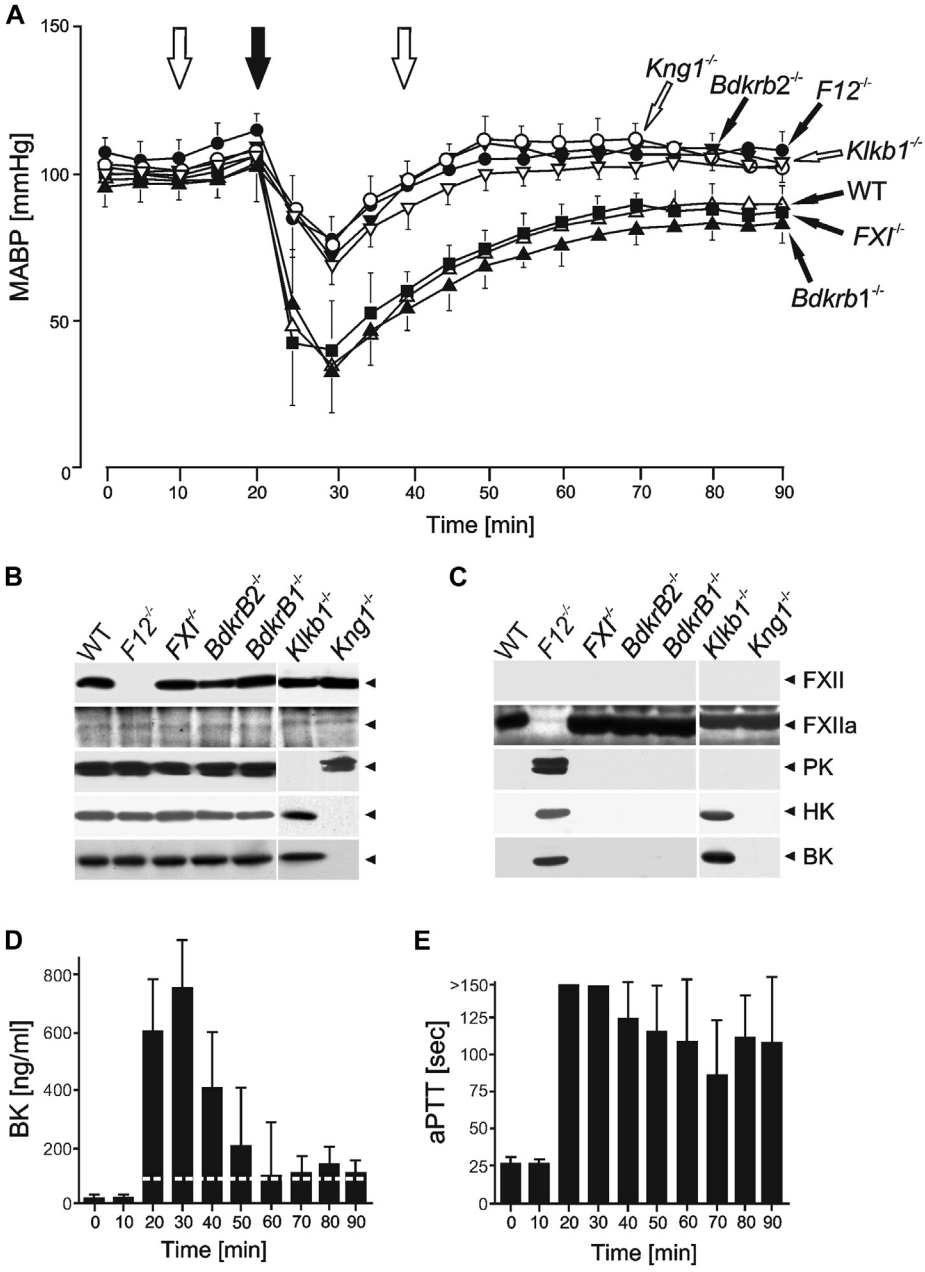

FIG 1. The bradykinin *(BK)*–forming contact system is activated in anaphylactic mice. Mice were intravenously injected with IgE-DNP and challenged 24 hours later with DNP-HSA injection. **A,** Arterial blood pressure during IgE/antigen-induced systemic anaphylaxis was measured in the left carotid artery in *F12*$^{-/-}$ *(solid diamonds)*, *FXI*$^{-/-}$ *(solid squares)*, *Klkb*$^{-/-}$ *(inverted open triangles)*, *Kng1*$^{-/-}$ *(open circles)*, *Bdkrb1*$^{-/-}$ *(solid triangles)*, *Bdkrb2*$^{-/-}$ *(solid circles)*, and WT *(open triangles)* mice. *Solid arrows* and *open arrows* indicate the time of DNP-HSA infusion and plasma sample collection, respectively. Means ± SDs of 10 mice per group are shown. **B** and **C,** Immunoblot analyses in plasma samples of IgE/antigen-challenged mice before (Fig 1, *B*) and after (Fig 1, *C*) DNP-HAS infusion with antibodies against FXII zymogen (nonprocessed single-chain FXII, αFXII, *upper row*), FXIIa, PK zymogen, HK, and the bradykinin sequence in HK (BK). **D** and **E,** Bradykinin levels were measured by using ELISA (Fig 1, *D*) and aPTT (Fig 1, *E*) in WT mouse plasma samples. Means ± SDs (n = 5) are shown.

Pharmacologic inhibition of FXII activity and B2R signaling interferes with anaphylaxis-induced systemic hypotension

Because an inherited deficiency of either FXII, PK, HK, or B2R attenuates the manifestations seen in allergic reactions, we proposed that pharmacologic targeting of bradykinin formation or its downstream signaling should confer similar protection from hypotension during acute episodes of anaphylaxis. Pretreatment of WT mice with a recombinant FXIIa inhibitor or an active PK inhibitor, rHA-infestin-4 and DX-88, respectively, largely reduced IgE/antigen-provoked decreases in MABP by about 50% compared with values in buffer-treated control animals (23 ± 9 and 26 ± 10 vs

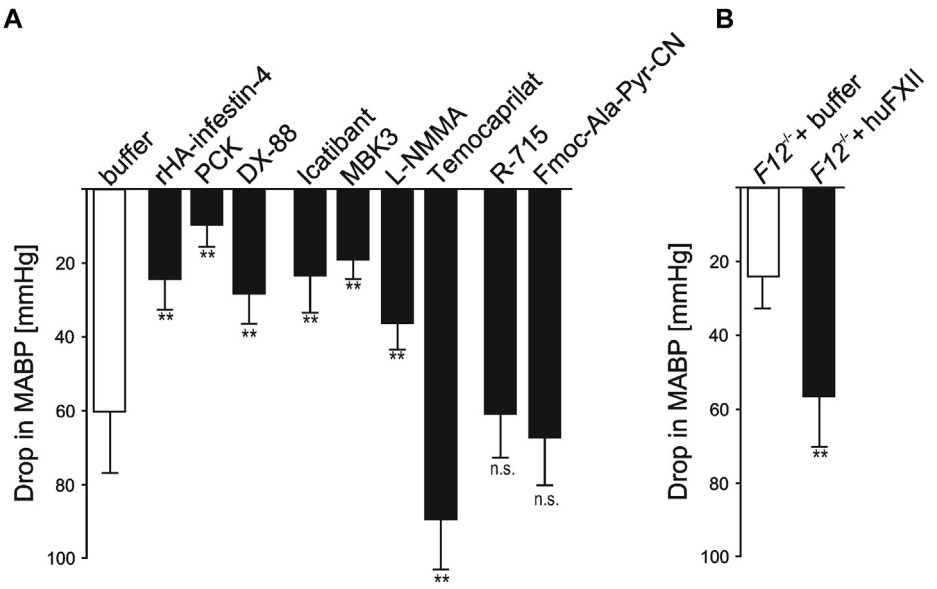

FIG 2. Pharmacologic inhibition of bradykinin formation and signaling interferes with blood pressure in a model of systemic anaphylaxis. **A,** WT or *F12*$^{-/-}$ mice were intravenously infused with IgE-DNP and challenged 24 hours later by means of application of DNP-HSA. Five minutes before allergen injection, WT animals were treated with the FXIIa inhibitors rHA-infestin-4 (15 mg/kg body weight) and PCK (8 mg/kg body weight), the PK inhibitor DX-88 (2 mg/kg body weight), the B2R antagonist icatibant (175 μg/kg body weight), the bradykinin blocking antibody MBK3 (900 mg/kg body weight), the NO synthase inhibitor N^G-monomethyl-L-arginine (*L-NMMA*, 300 mg/kg body weight), the ACE inhibitor temocaprilat (10 mg/kg body weight), the B1R antagonist R-715 (5 mg/kg body weight), and the PRCP inhibitor Fmac-Ala-Pyr-CN (100 mg/kg body weight) or buffer vehicle. **B,** *F12*$^{-/-}$ mice were intravenously reconstituted with buffer or human FXII (2 mg/kg body weight) 5 minutes before challenge. The maximum decrease in central arterial blood pressure 10 minutes after challenge is shown. Means ± SDs of 8 mice in Fig 2, *A*, and 6 mice in Fig 2, *B*, are shown. $^{**}P < .05$ vs buffer, unpaired Student *t* test. *n.s.*, Not significant.

60 ± 19 mm Hg; Fig 2, *A*). PCK, which blocks both FXII and PK activity,[22] largely blunted the hypotensive responses in our systemic anaphylaxis model. Consistently, the B2R antagonist icatibant and the anti-bradykinin mAb MBK3, which interferes with FXIIa-initiated HK processing in plasma,[23] protected against IgE/antigen-driven adverse effects. Nitric oxide (NO) is a major intracellular mediator of bradykinin signaling that regulates vascular tone.[29] The NO synthase inhibitor N^G-monomethyl-L-arginine monoacetate interfered with anaphylaxis-induced decreases in MABP (37 ± 8 mm Hg). Angiotensin-converting enzyme (ACE) degrades bradykinin and also converts angiotensin I to angiotensin II.[30] Use of the ACE inhibitor temocaprilat reverses protection from IgE/antigen-induced hypotension. Targeting the B1R with a selective inhibitor, R-715, did not interfere with allergen-stimulated decreases in MABP. Prolylcarboxypeptidase (PRCP) has been identified as an alternative PK activator. PRCP inhibition with Fmoc-Ala-Pyr-CN did not affect hypotensive responses either. Results with the pharmacologic inhibitors are consistent with results obtained in gene-deleted mice and reveal a critical role for the bradykinin-producing contact system in systemic anaphylaxis in mice.

To analyze the role of human FXII for blood pressure regulation in anaphylaxis, we reconstituted *F12*$^{-/-}$ mice with human FXII protein. Intravenous infusion of human FXII protein (2 μg/g body weight) normalized the prolonged aPTT of treated animals (27 ± 7 seconds) and restored susceptibility for IgE/antigen-triggered hypotension. The decrease in MABP in human FXII protein–reconstituted *F12*$^{-/-}$ mice was similar to that seen in WT mice (57 ± 17 mm Hg; Fig 2, *B*).

Kininogen cleavage as a biomarker of contact system activation in patients

Our murine data suggested that the contact system is operative during conditions of mast cell activation and that modulation of its activity represents an alternative approach in the management of anaphylaxis reactions. Because bradykinin is rapidly degraded (plasma half-life, <30 seconds[31]), investigations next determined a more stable assay for measuring contact system activation. We established a sensitive Western blot–based assay that quantifies the degree of HK cleavage to assess contact system activation in patients' plasma (Fig 3, *A*). We took advantage of the fact that the bradykinin-containing single-chain form of HK migrates at an apparent molecular mass of 118 kDa in reduced SDS-PAGE. Bradykinin liberation from its precursor produces a 55-kDa HK light chain fragment. Anti-HK antibody I108 detects single-chain HK and produces a faint light chain signal in freshly drawn citrated human plasma. Plasma collected into sodium citrate supplemented with protease inhibitors (that block contact system proteases) did not increase single-chain HK signaling further (Fig 3, *A*, lane 1). High-molecular-weight dextran sulfate (500,000 Da) is a strong FXII contact activator.[32] Incubation of plasma with dextran sulfate (25 μg/mL) for 20 minutes induced complete cleavage of plasma HK (lane 14). We quantified the single-chain HK signal by using a densitometric scan of a mixture of untreated (100% HK) and dextran sulfate–activated plasma (0% HK) and plotted the data by a third-order Bezier curve (B0: 0.9601; B1: 0; B2: 0.007125 x^2; B3: 7.311×10^{-5} x^3; Fig 3, *B*). To confirm that HK cleavage correlates with bradykinin production, we

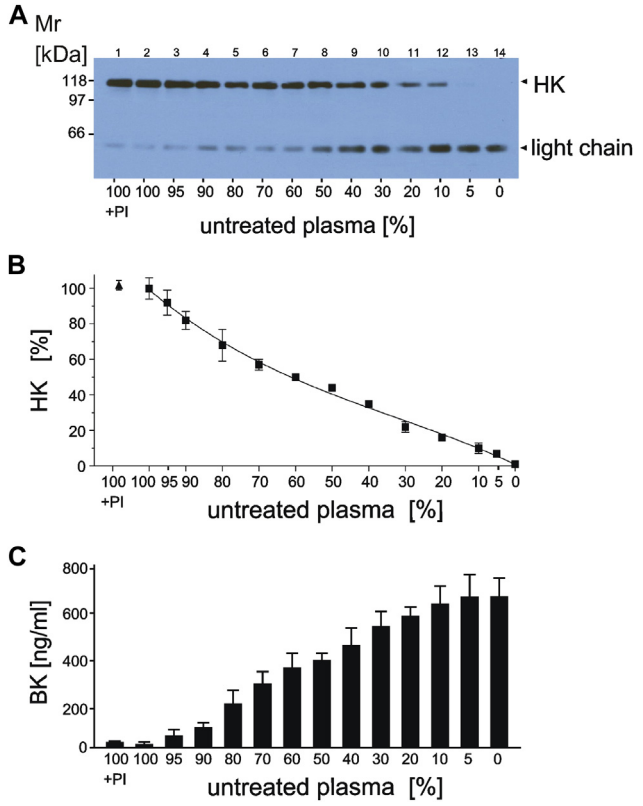

FIG 3. HK cleavage functions as a plasma biomarker to assess contact system activation. **A,** Human plasma was incubated with the contact system activator dextran sulfate (500,000 Da) for 20 minutes at 37°C to trigger complete cleavage of plasma HK. Mixtures of dextran sulfate–treated and nontreated plasma were probed for HK by means of Western blotting with the anti-HK heavy chain antibody I108. A representative photographic film (n = 10) is shown. HK in plasma samples that were directly collected into a protease inhibitor cocktail (+PI) in the absence of dextran sulfate served as a control. **B,** Densitometric scans assess HK signal intensity in the plasma mixture. HK signal in untreated plasma supplemented with protease inhibitors was set to 100%. Means ± SDs (n = 10 mice) are shown. **C,** Bradykinin *(BK)* in mixtures of dextran sulfate–treated and untreated plasma samples was determined by means of ELISA. Means ± SDs (n = 5) are shown. *Mr,* Relative molecular mass.

measured bradykinin levels in the mixture using ELISA (Fig 3, *C*). The samples were supplemented with ACE, aminopeptidase P, and the carboxypeptidase N inhibitors enalaprilat (130 nmol/L), apstatin (N-[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-PPA-NH2), and Plummer inhibitor (DL-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid), respectively, before dextran sulfate addition to block the metabolism of produced bradykinin. Bradykinin levels were inversely related to the HK signal and were high (>750 ng/mL) in samples with undetectable HK levels. Quantification of HK is challenging because of preanalytic artificial FXII activation. We noted that repetitive freeze-thaw cycles triggered complete HK cleavage in plasma (see Fig E3 in this article's Online Repository at www.jacionline.org). In contrast, storage at room temperature for 6 hours at 4°C for 24 hours or −20°C for up to 6 months, respectively, had no detectable effect on HK consumption. Plasma samples stored at −20°C for less than 3 months and thawed once shortly before analysis were used for all of our studies.

HK cleavage in patients with anaphylaxis

We enrolled 10 patients, 4 men and 6 women aged 20 to 79 years (median age, 40 years; IQR, 31.8-47.8 years), who were admitted to our hospital with anaphylaxis. Table I summarizes their clinical data and the times of sampling after symptom onset. Anaphylaxis was diagnosed and classified according to the 2006 National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network criteria.[18] Four patients presented with severe anaphylaxis, 4 patients presented with moderate B symptoms, and 2 patients presented with moderate A anaphylaxis. Patient symptomatology varied: the most common presenting manifestation was a skin reaction, such as pruritus and urticaria (10/10). Gastrointestinal signs included nausea, vomiting, and diarrhea (5/10); respiratory symptoms included airway edema causing dyspnea or stridor (5/10); and cardiovascular symptoms included dizziness, weakness, pulse abnormalities, and hypotension (4/10). One patient was recently prescribed an ACE inhibitor for his hypertension, and none were receiving treatment with immunosuppressive agents. Medications induced anaphylaxis in 5 patients; food was the triggering factor in 4, and anisakid nematodes were the triggering factor in 1 patient, respectively. We analyzed single-chain HK levels in patient plasma at admission, during anaphylaxis (30 minutes to 10 hours after onset of symptoms), and at basal conditions (>2 weeks after the anaphylaxis). In 3 patients (patients 1-3) we collected 2 consecutive samples during the admission process. For comparison, we used plasma samples of 10 age- and sex-matched healthy control subjects (4 atopic and 6 nonatopic subjects).

Plasma was probed with I108 antibody (Fig 4, *A*), and HK signal was quantified by means of densitometric scanning (Fig 4, *B*). HK was largely reduced to 33.7% (IQR, 20% to 45%; 95% CI, 22.75% to 53.63%) in all samples of patients with anaphylaxis compared with 97.5% HK levels in basal conditions (IQR, 95% to 100%; 95% CI, 93.7% to 99.9%; $P < .001$; Fig 4, *C*). Plasma HK levels in patients 1 to 3 showed that the bradykinin precursor was consumed within the first hours of symptom development. There was no statistically significant difference between plasma HK levels in patients at basal conditions compared with those seen in healthy control subjects ($P > .05$). Plasma HK levels were negatively associated with the severity of anaphylactic reactions and significantly lower in patients with severe anaphylaxis (18.4%; IQR, 2.5% to 27.5%; 95% CI, 0.5% to 35.6%) compared with those in patients with moderate anaphylaxis (45% [IQR, 32.3% to 61%] in the moderate B group and 38% [IQR, 31.5% to 40.5%] in the moderate A group, $P < .01$; Fig 4, *D*). In contrast to the decrease in plasma HK levels during anaphylaxis, there was no decrease in plasma HK levels in patients with mastocytosis or idiopathic histaminergic angioedema, respectively (see Fig E4 in this article's Online Repository at www.jacionline.org).

Mast cell–mediated contact system activation in anaphylaxis

The murine anaphylaxis models (Fig 1) critically depend on IgE/antigen-stimulated mast cells that release their granule contents with heparin as a major component. Mast cell heparin polysaccharide is a potent FXII contact activator in mouse models[12] and human plasma,[11] inducing contact system–mediated bradykinin formation in plasma.[10] We analyzed the effect of heparin on contact system activation in plasma samples

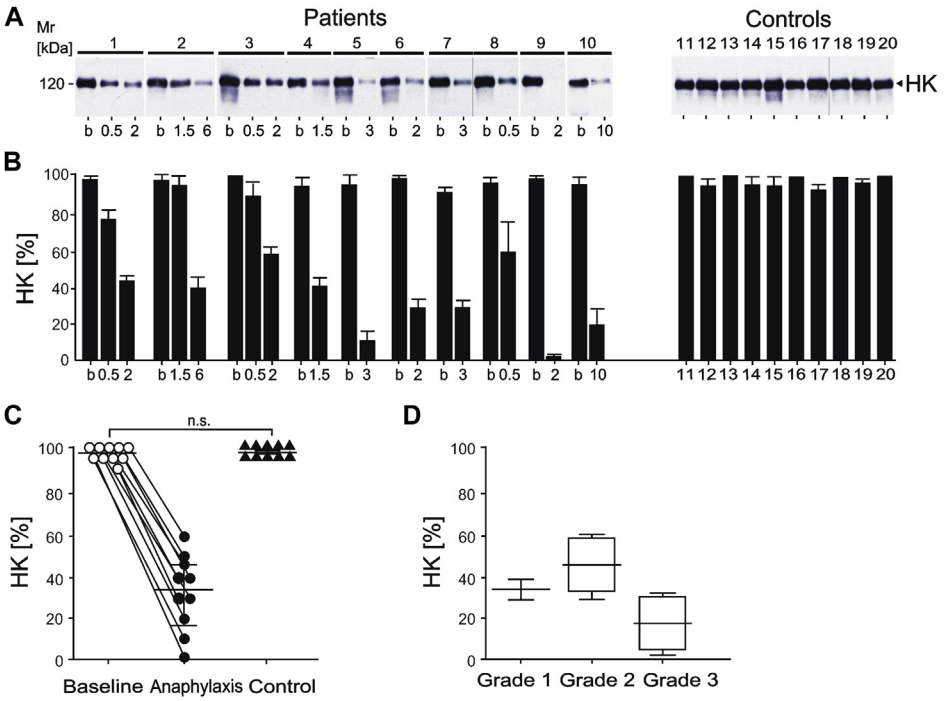

FIG 4. HK is consumed during anaphylaxis in patients. **A,** Plasma samples were collected from 10 patients with different grades of anaphylaxis at the indicated time points after symptom onset and at baseline (>14 days after anaphylaxis episode). For patients 1 to 3, 2 consecutive samples at an early and later time point from the onset of anaphylaxis were obtained. Plasma samples from 10 age- and sex-matched healthy control subjects were collected, and these served as controls. Plasma samples (0.25 μL each) were resolved under reducing conditions on 10% SDS-PAGE and probed for HK by means of Western blotting with I108 antibody. Time points of sample collection are given below the photographic film. Samples collected in basal conditions are designated with *b*. A representative film (n = 5) is shown. **B,** HK signal intensity was assessed by using densitometric scans. Means ± SDs (n = 5) are shown. **C,** Baseline *(open circles)* and HK levels from patients with anaphylaxis at the time of admission *(solid circles)*. Plasma HK in matched control subjects *(solid triangles)*. **D,** Plasma HK levels are blotted for each grade of anaphylaxis. *Grade 1*, Moderate A; *Grade 2*, moderate B; and *Grade 3*, severe anaphylaxis. Medians with IQRs and box-and-whisker plots are shown. *Mr*, Relative molecular mass.

of 10 patients admitted with an anaphylactic episode (Fig 5, *A* and *B*). FXII and PK zymogen levels during anaphylaxis were reduced to 2% to 65% of basal levels (see Fig E5 in this article's Online Repository at www.jacionline.org), with the latter being similar to those observed in healthy control subjects (92.7% ± 7.9% vs 96.0% ± 4.7%). Decreases in plasma FXII and PK zymogen levels paralleled each other and were highest in those patient samples with the largest decrease in HK levels (eg, patients 5, 6, 7, and 9). Plasma levels of both FXII and PK negatively associated with the severity of anaphylactic reactions and were significantly lower in patients with severe anaphylaxis (20% [IQR, 10% to 35%; 95% CI, 10% to 40.5%] and 15% [IQR, 10% to 57.5%; 95% CI, 5% to 80%] for FXII and PK, respectively) compared with those seen in patients with moderate anaphylaxis (FXII: 22.5% [IQR, 12.5% to 32.5%] in grade 2 and 30% [IQR, 15% to 45%] in grade 1 [$P < .05$; Fig 5, *C*]; PK: 30% [IQR, 28.5% to 57.5%] in grade 2 and 32.5% [IQR, 10% to 55%] in grade 1 [$P < .05$; Fig 5, *D*]). The anti-Xa activity assay is a sensitive measure of plasma heparin activity and commonly used in the clinical setting for monitoring heparin plasma levels.

Anti-Xa activity was more than 4-fold higher in patients' plasma during an anaphylaxis attack compared with levels in basal conditions (4.0 IU/mL [IQR, 2.5-5.5 IU/mL; 95% CI, 2.48-6.706 IU/mL] vs 1 IU/mL [IQR, 0.25-1.75 IU/mL; 95% CI, 0.1784-2.322 IU/mL], $P < .05$), indicating significant heparin release (>5 μg/mL plasma). Anti-Xa levels did not significantly differ in patients at basal conditions compared with those seen in healthy control subjects (Fig 5, *E*) and were highest in patients with severe anaphylaxis (Fig 5, *F*).

Mast cell granules release tryptase, which is used as a diagnostic biomarker of mast cell activation in anaphylaxis. Patients' serum tryptase levels were increased during anaphylaxis compared with those in basal conditions and were positively correlated with plasma HK cleavage ($P = .0002$, Spearman $R = 0.7$; see Fig E6 in this article's Online Repository at www.jacionline.org), supporting the hypothesis that mast cell degranulation is associated with bradykinin formation. Together, the data show that during anaphylaxis, heparin has the capacity to trigger bradykinin formation through activation of the FXII-driven contact system.

DISCUSSION

For more than a century, IgE-mediated systemic hypersensitivity reactions have been shown to be associated with abnormalities in blood coagulation.[33,34] Allergen-initiated coagulation defects are transient and short lived.[35] aPTT, a measure of the FXIIa-driven intrinsic coagulation pathway, is significantly

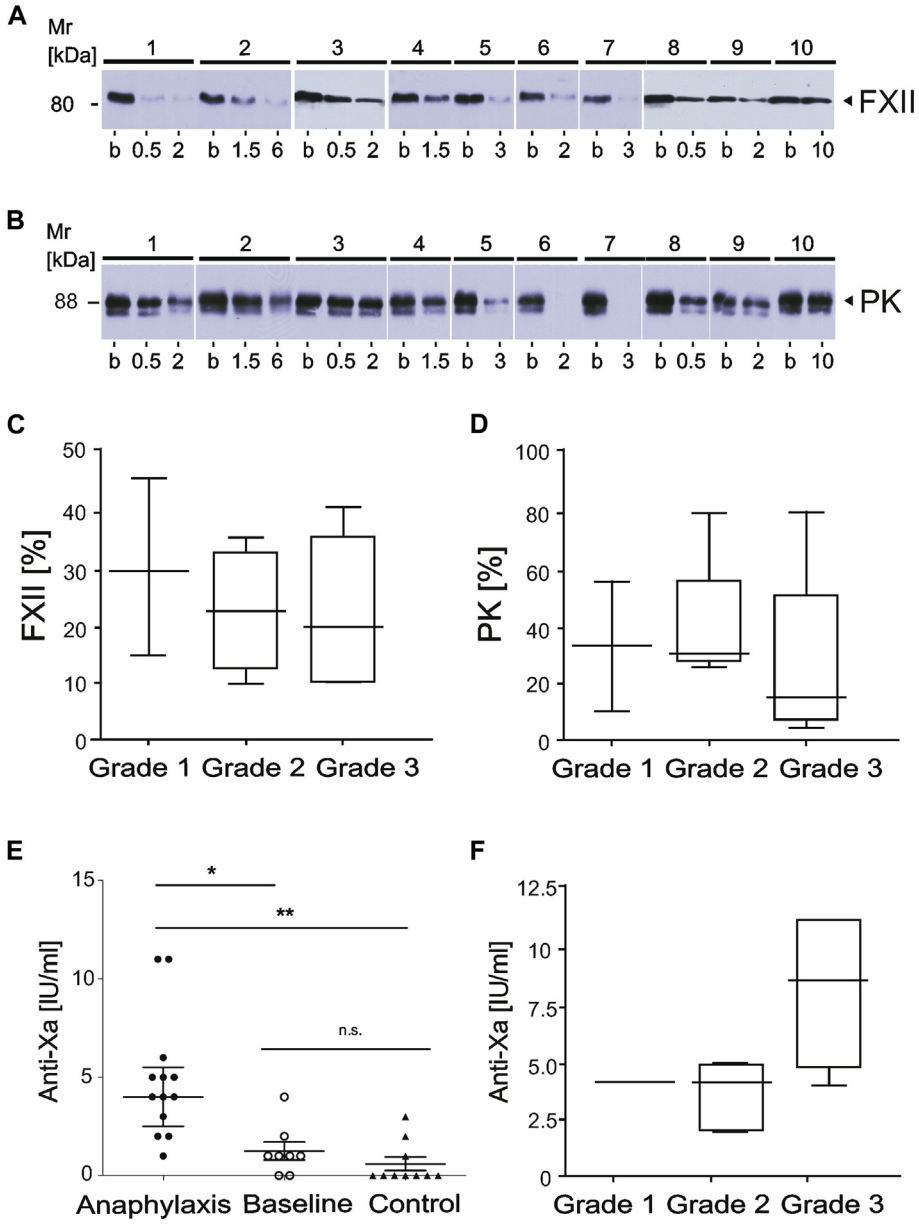

FIG 5. The contact system is activated in patients with anaphylaxis. **A** and **B,** Plasma samples from patients with anaphylaxis collected at indicated time points after the onset of symptoms and at baseline, as described in Fig 4, were probed for zymogen forms of FXII (Fig 5, *A*) and PK (Fig 5, *B*) by using Western blotting. **C, D,** and **F,** Plasma FXII (Fig 5, *C*), PK zymogen (Fig 5, *D*), and anti-Xa (Fig 5, *F*) levels were blotted for each grade of anaphylaxis. *Grade 1,* Moderate A; *Grade 2,* moderate B; and *Grade 3,* severe anaphylaxis. Medians with IQRs and box-and-whisker plots are shown. **E,** Anti-Xa as a measure of plasma heparin was analyzed in patients' samples after the onset of anaphylaxis at admission to the hospital and at baseline.

prolonged in plasma samples of patients with anaphylaxis. In contrast, prothrombin time, which assesses the extrinsic pathway of coagulation, is intact in patients with insect toxin– or food-triggered anaphylaxis.[36,37] Consistently, in a rabbit model of passive systemic anaphylaxis, plasma levels of the contact pathway factors were largely reduced and a significant prolongation of the aPTT was observed, suggesting that IgE/antigen-triggered mechanisms can activate directly or indirectly the intrinsic blood coagulation system *in vivo*.[38] In contrast to impaired fibrin formation in *ex vivo* coagulation tests, patients at large do not exhibit clinical signs of bleeding during anaphylaxis.[36] Indeed, a hemostatic defect is not a typical manifestation of anaphylactic reactions.[39,40] Our study offers a rationale for the intriguing relationship between the blood coagulation system and systemic hypersensitivity responses. Heparin levels increase during anaphylaxis, and immunoprint analyses reveal that the FXII-driven contact system becomes activated, as seen by the proteolytic processing and consumption of contact factors in the plasma of both experimental animals (Fig 1) and patients (Figs 4 and 5).

The schematic presentation summarized the findings of our study in the context of anaphylaxis (Fig 6). Using murine models,

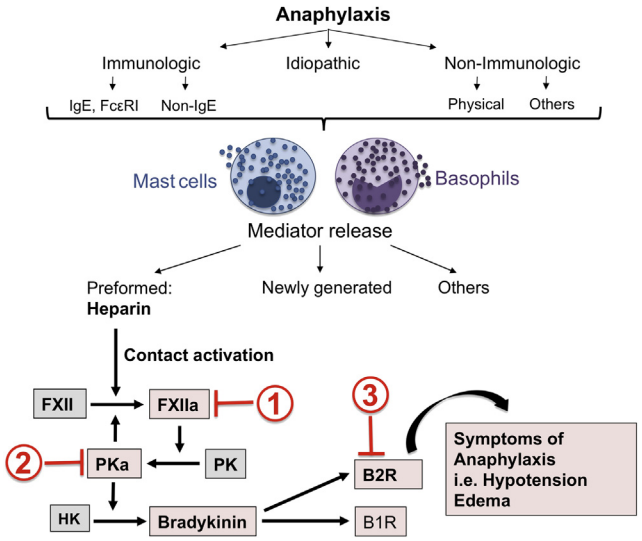

FIG 6. Scheme of the proposed role of the contact system in patients with anaphylaxis. Anaphylaxis is commonly mediated through immune IgE-dependent mechanisms. Additionally, nonimmunologic or idiopathic mechanisms exist. Anaphylaxis triggers cumulatively lead to activation of mast cells and basophils with mediator release. The glycosaminoglycan heparin initiates FXII contact activation that in turn activates its PK zymogen to the active protease *(PKa)*, which liberates the peptide hormone bradykinin from its precursor HK). Bradykinin binding to B2Rs, but not B1Rs, triggers symptoms of anaphylaxis. This study identifies 3 potential targets for pharmacologic inhibition of contact system–mediated symptoms of anaphylaxis: (1) inhibitors of FXIIa (eg, rHA-infestin-4 and PCK); (2) inhibitors of PK (eg, DX-88); and (3) inhibitors of B2R (eg, icatibant), PK-mediated BK formation (MBK3), or B2R signaling (N^G-monomethyl-L-arginine monoacetate *[L-NMMA]*).

our laboratory has shown that IgE/antigen-stimulated mast cells release heparin locally, which induces bradykinin formation in a FXII-dependent manner. The implications of this process account for the manifestations of increased vascular permeability.[12] A case report associated wasp sting–induced anaphylaxis with a decrease in plasma HK levels and some decrease in plasma PK levels with normal FXII activity.[41] HK levels were also reduced in patients with severe anaphylaxis induced by insect venom hypersensitivity,[42] whereas FXII levels remained within the normal range.[40,43] In these studies plasma FXII activity was determined by using an aPTT-based clotting assay, which has variable sensitivity and by principle does not become prolonged unless factor activity decreases to less than 20%. In our study only 2 patients (patients 5 and 9) had a prolonged aPTT of greater than 100 seconds and the highest anti-Xa (a measure of plasma heparin) of 11 U/mL concomitant with high HK cleavage (Fig 4). In sharp contrast to deficiencies in other proteins of the coagulation system, such as factors VIII and IX, patients with a deficiency in FXII, PK, and HK have a completely normal hemostatic capacity.[44] Activated mast cells release heparin into the circulation. In addition to consumption of contact system proteins, the polysaccharide prolongs the aPTT through antithrombin III–dependent mechanisms; this anticoagulant activity might account for the rare bleeding episodes seen in a few patients with anaphylaxis.[45]

Bradykinin has been proposed to play a role in anaphylaxis for decades; however, a convincing mechanism for its generation in shock was lacking. Infusion of the *ex vivo*–generated active fragment of FXII, which contains the enzymatic domain, depletes circulating plasma prekallikrein levels and induces hypotensive reactions both in healthy subjects and rat models. The fragment of FXII–triggered hypotensive episodes are insensitive to COX inhibition (indomethacin) but largely impaired in the absence of PK, indicating that bradykinin (not prostaglandins) is the main mediator of systemic manifestations, including rapid decreases in systemic blood pressure in a rat model.[46] Determining plasma bradykinin levels is a challenging task and technically limited, primarily because of its rapid metabolism by multiple endopeptidases and exopeptidases.[47] Additionally, FXII is susceptible to activation by artificial surfaces, including exposure of whole blood during collection, and FXIIa might initiate bradykinin production preanalytically (reviewed in Maas and Renné[48]). As such, and because of patient sampling occurring in an emergency department, we were unable to determine bradykinin levels.

Kininases degrade bradykinin, with kininase I (carboxypeptidase N) being a potent inactivator of bradykinin *in vivo*. Removal of the C-terminal arginine residue from bradykinin results in des-Arg^9-BK that no longer binds to the B2R. des-Arg^9-BK is still able to activate the B1R and induces vasodilation similar to the effects seen with B2R signaling.[49] Systemic hypotension during LPS-triggered endotoxemia in mice is predominantly mediated through B1R signaling, with minor contributions from the B2R.[50] The B2R is constitutively expressed by various vascular cell types, such as endothelial cells, vascular smooth muscle cells, and cardiac myocytes.[51] In contrast, B1R expression is inducible and the receptor is virtually absent under physiologic conditions; however, expression is largely upregulated in inflammation by cytokines such as IL-1β. The B1R plays an important role in hypotension and bronchoconstriction during chronic inflammatory states[50] yet has a minor contribution in acute hypotensive reactions, such as those observed during anaphylaxis (Figs 1 and 2). Anaphylactic shock is primarily considered a form of distributive shock characterized by a profound reduction in vascular tone. Generation of NO by G(q)/G(11)-mediated signaling is central to the development of hypotension during anaphylaxis.[52] The B2R is coupled to G(q) in the endothelium and induces NO-mediated vasodilation. Consistent with our data in murine anaphylaxis, targeting the B2R and its downstream signaling protects from anaphylaxis reactions in sheep,[52] guinea pigs,[53] and rats.[49] *Vice versa*, patients receiving ACE inhibitor therapy are at increased risk for anaphylactic reactions. ACE inhibitors increase plasma bradykinin levels and correlate with a higher degree of anaphylaxis in experimental models in sheep.[54] Plasma HK levels are the sum of consumption and secretion of newly synthesized proteins. Patient 10 presented a severe anaphylaxis; however, contact system levels in plasma samples drawn 10 hours after the onset of symptoms were relatively high. *De novo* protein synthesis and release from intracellular stores within the 10-hour period offer a rational for the observed high plasma levels of the contact factors in that specific patient.

Increased bradykinin plasma levels were observed at the initial phase of acute hypersensitivity reactions; however, the mechanisms driving bradykinin formation and its importance in anaphylaxis have remained enigmatic. Purified human lung mast cells release mediators with kininogenase activity in an IgE-dependent manner, such as tryptase.[7,55] However, the unique pH optimum of tryptase functioning as a serine protease and slow kinetics of the HK cleavage reaction raise doubts about the physiologic significance of tryptase-mediated HK processing. FXIIa-independent modes of generating bradykinin from HK *ex vivo* involve heat shock protein

90[56] and PRCP[57]; however, their *in vivo* relevance remains to be established. In our mouse anaphylaxis models the PRCP inhibitor Fmoc-Ala-Pyr-CN did not attenuate decreases in blood pressure (Fig 2). Heparin activates FXII in human plasma, and minute amounts of heparin (≥4 μg/mL) are sufficient to induce contact-mediated autoactivation of plasma FXII *ex vivo*.[12] Heparin also protects FXIIa from inhibition by C1 esterase inhibitor (C1INH).[58] FXIIa processes its substrate, PK zymogen; however, the homologous plasma protein FXI is not activated under these circumstances, suggesting the presence of a regulatory mechanism for plasma kallikrein–directed activity of FXIIa. A rationale for selective FXIIa-mediated PK activation is still speculative but can be attributed to the nature of negatively charged surfaces that are exposed by the misfolded proteins.

Additionally, different FXIIa forms that occur in activation reactions might also contribute to selective bradykinin formation.[59] Formation and activities of the peptide hormone are tightly controlled at various levels. HK binding to proteoglycans regulates local bradykinin generation, which leads to characteristic circumscribed swelling (eg, in patients with angioedema). Circulating bradykinin is rapidly cleared in the lung by kininases before reaching pressure resistance vessels that regulate blood pressure.[60] Furthermore, various genetic variations in the kallikrein-kinin system affect vascular bradykinin effects, including mutations in the bradykinin-degrading enzymes[61] and in the bradykinin receptors.[49,62] HK cleavage was moderately correlated with increased tryptase levels, reflecting the complex pathology of anaphylaxis involving basophils[63] and great variability of the biomarker tryptase in patients with anaphylaxis.[19] Mouse models have demonstrated that basophils are dispensable for IgE-mediated anaphylaxis but play a crucial role in IgG-mediated anaphylaxis.[64] In contrast, the effect of basophils for anaphylaxis in patients seems more complex. Basophils contribute to food ingredient–triggered anaphylaxis.[63,65] Indeed, patients with low tryptase levels in our study were mostly allergic to food components.

The function of mast cell–released heparin as an initiator of contact activation–mediated bradykinin formation in patients with anaphylaxis is reminiscent of reports that associated therapeutic heparin infusion and contact system activation in a series of life-threatening complications. At the end of 2007, there was a dramatic increase in heparin-induced adverse reactions in the United States and Germany, such as lethal acute hypersensitivity reactions in patients receiving commercially available intravenous heparin of specific lots from a single manufacturer. Conservatively, more than 150 patients died from anaphylactic hypotension associated with intravenous heparin treatment. Comprehensive analyses identified an unnatural contaminant occurring in suspect preparations of heparin that was characterized as oversulfated chondroitin sulfate.[66] Oversulfated chondroitin sulfate–contaminated heparin has a greatly increased potency for activating FXII and triggering PK-mediated bradykinin formation in human plasma and in a model of experimental hypotonic shock *in vivo* compared with heparin.[67] These catastrophic anaphylactic reactions in patients are analogous to experimental hypotensive shock models induced by dextran sulfate–stimulated bradykinin formation in pigs. Infusion of dextran sulfate induced transient systemic hypotension, and the B2R antagonist HOE140 (icatibant) blocked this effect on blood pressure,[68] suggesting the therapeutic value of this agent in human anaphylaxis. Icatibant has recently been approved for the treatment of a rare inherited disease, hereditary angioedema (HAE), that is clinically characterized by recurrent life-threatening acute swelling episodes affecting the skin, oropharyngeal, laryngeal, or gastrointestinal mucosa resulting from increased vascular permeability. The mechanisms that result in increased vessel leak in patients with HAE are controversial; however, excessive bradykinin formation caused by pathologic activation of the FXII-driven contact system is a consistent finding during acute episodes. HAE develops in subjects who are quantitatively or qualitatively deficient (HAE type I and II, respectively) in C1INH, the endogenous inhibitor of FXIIa and PK.[69] C1INH deficiencies facilitate the excessive activation of the FXII-driven contact system cascades and the development of edema in patients with HAE.[70] Consistent with animal models,[71] clinical studies have confirmed the excessive contact system–mediated bradykinin generation in C1INH-deficient subjects[72] and identified bradykinin as the principal mediator of vascular leakage in HAE-related swelling attacks in patients.[60] The anti-FXIIa antibody 3F7 has been shown to interfere with FXIIa-driven clotting in cardiopulmonary bypass systems (extracorporeal membrane oxygenation).[73] The recombinant antibody is humanized and inhibits dextran sulfate– and polyphosphate-triggered FXII contact activation,[74] suggesting therapeutic use of 3F7 in patients with angioedema and anaphylaxis.

In addition to anaphylaxis and HAE, contact system–mediated bradykinin participates in a variety of allergic and inflammatory disease states, including bacteremia and sepsis,[75] vasculitis,[76] rhinitis,[13] and possibly asthma.[77] The main limitation in our study is the small sample size, and there is a need for larger clinical studies to analyze the degree of contact system activation in these disease states and various allergic disorders that are associated with aberrant mast cell and basophil activity. A recent clinical study has shown that multiple inflammatory pathways drive reaction severity in patients with anaphylaxis; however, the contact system was not analyzed in these patients.[3] By using the immunoblot-based techniques established in the current article, these analyses could be an attractive goal for future clinical trials. Cumulatively, the current study shows that heparin-triggered activation of the bradykinin-forming contact system is operative in patients with anaphylaxis. Targeting bradykinin interferes with hypersensitivity reactions in mouse models. The contact system is conserved among human subjects and mice, suggesting that targeting bradykinin generation or its downstream signaling is a promising strategy for interfering with anaphylaxis and possibly other allergic diseases.

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Key messages

- **The FXII-driven contact system contributes to anaphylaxis in patients and mice.**
- **The severity of anaphylaxis is associated with the intensity of contact system activation.**
- **Targeting mast cell–initiated contact system activation offers novel therapeutic strategies for interference with anaphylaxis.**

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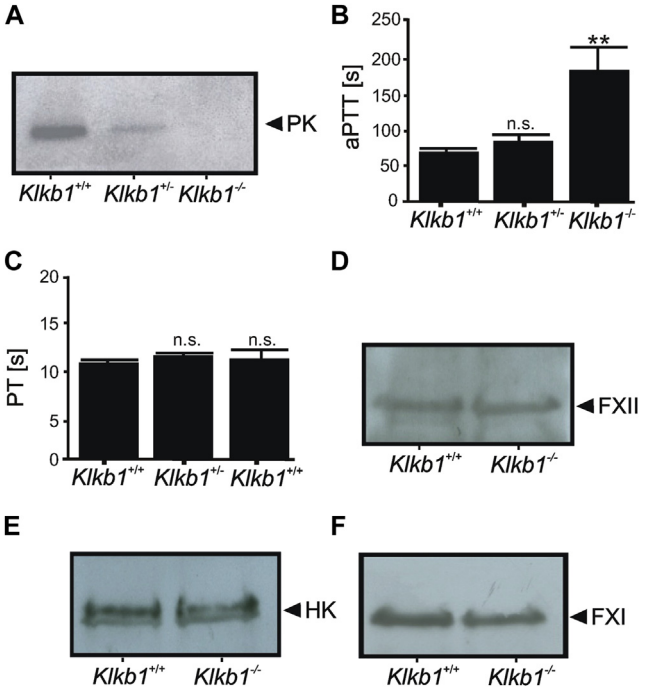

FIG E1. Contact system proteins in PK-deficient mice. Western blots of plasma from WT (*Klkb1*$^{+/+}$) mice and animals that are heterozygous (*Klkb1*$^{+/-}$) or homozygous (*Klkb1*$^{-/-}$) deficient in *Klkb1* gene expression. Plasma (0.2 μL per lane) was separated under reducing conditions by using primary antibodies recognizing the contact proteins PK **(A)**, FXII **(D)**, HK **(E)**, and FXI **(F)**. The *arrowheads* point to PK, FXII, HK, and FXI, respectively. The aPTT (a measure of contact system driven coagulation; **B**) and prothrombin time (*PT*; a measure of tissue factor–driven coagulation; **C**) were analyzed in PK-deficient mouse plasma. Means ± SEMs (n = 5) are shown. *n.s.*, Nonsignificant. ***P* < .05 versus *Klkb1*$^{+/+}$, unpaired Student *t* test.

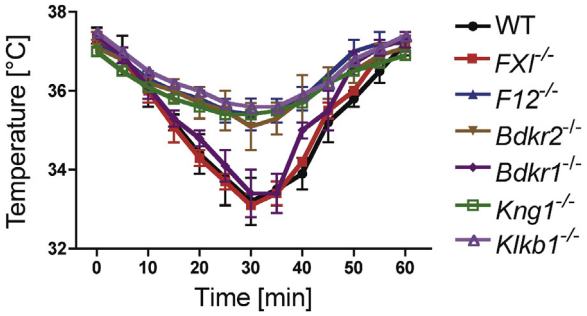

FIG E2. Deficiency in contact system proteins protects against allergen-induced hypothermia. In a model of passive systemic anaphylaxis, mice were intravenously injected with IgE and challenged at 24 hours by means of DNP-HSA infusion. Skin temperature after antigen challenge was analyzed in WT, *FXI*$^{-/-}$, *F12*$^{-/-}$, *Bdkrb2*$^{-/-}$, *Bdkrb1*$^{-/-}$, *Kng1*$^{-/-}$, and *Klkb*$^{-/-}$ mice.

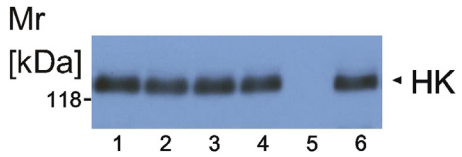

FIG E3. Repeated plasma freeze-thaw cycles induce HK cleavage. We tested the preanalytic conditions that can affect HK processing during sample preparation and determined plasma HK levels in various samples using Western blotting with I108 antibody. Plasma was collected directly into citrate *(1)* or citrate supplemented with protease inhibitors *(2)* and immediately frozen at −20°C. Citrated plasma was stored at room temperature for 6 hours *(3)* or at 4°C for 24 hours *(4)* and analyzed without freezing. Citrated plasma was frozen at −20°C, thawed 12 hours later, and frozen again at −20°C *(5)*. Samples collected into citrate supplemented with protease inhibitors and subjected to a freeze-thaw cycles as above *(6)*. *Mr*, Relative molecular mass.

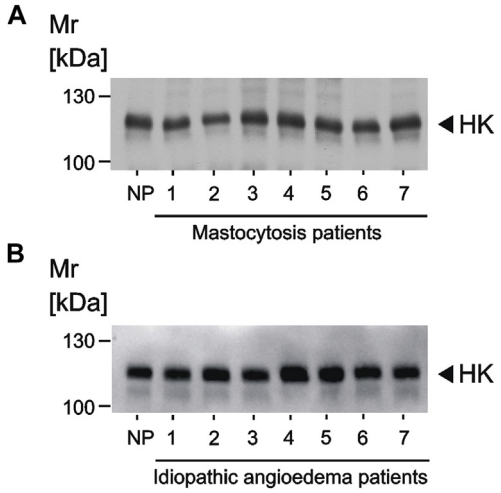

FIG E4. HK cleavage in patients with mastocytosis and idiopathic histaminergic angioedema. Mastocytosis **(A)** and idiopathic histaminergic angioedema **(B)** patient samples (0.25 μL per lane) were separated by using reducing SDS-PAGE and analyzed by using Western blotting for HK cleavage. Plasma of a healthy control subject *(NP)* served as a control. *Mr*, Relative molecular mass.

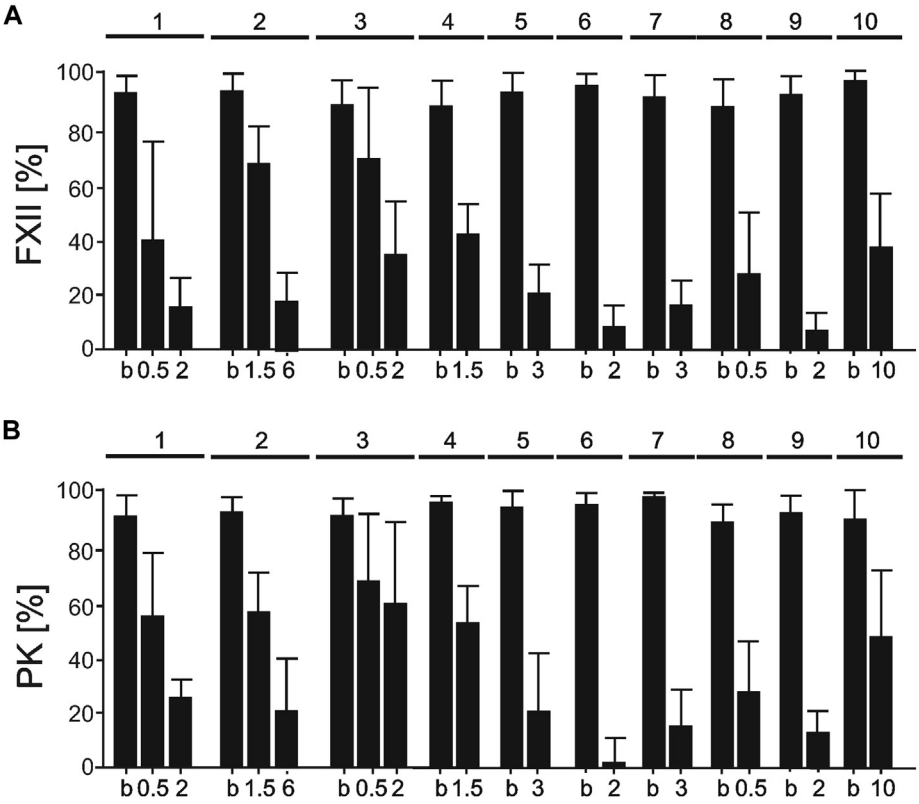

FIG E5. FXII and PK are consumed during anaphylaxis in patients. Relative FXII **(A)** and PK **(B)** zymogen antigen levels in plasma samples from 10 patients with different grades of anaphylaxis at indicated time points after the onset of symptoms and at baseline (designated with *b* >14 days after the anaphylaxis episode) are shown. For patients 1 to 3, 2 consecutive samples at an early and later time point from the onset of anaphylaxis were obtained. Plasma samples from 10 age- and sex-matched healthy control subjects were collected and served as controls. FXII and PK levels were assessed by using densitometric scans from the intensity of Western blots signals. Means ± SDs (n = 4) are shown.

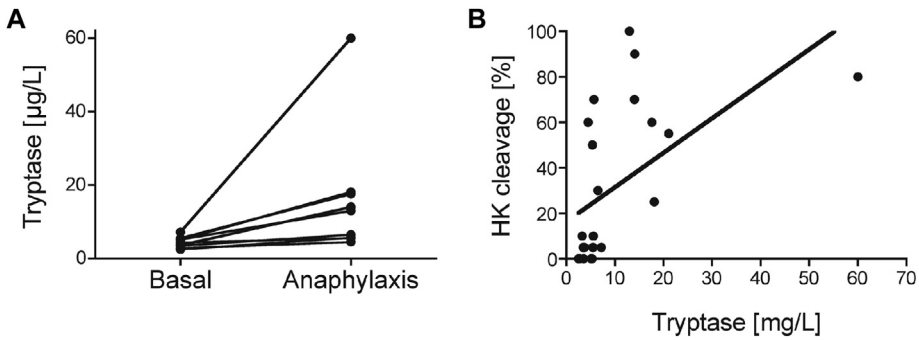

FIG E6. Correlation of HK cleavage and tryptase levels during anaphylaxis and at baseline. **A,** Serum tryptase levels from 10 patients at baseline and during acute anaphylaxis. **B,** Correlation between plasma HK cleavage and serum tryptase levels during anaphylaxis and at baseline.