# Factor XII–independent activation of the bradykinin-forming cascade: Implications for the pathogenesis of hereditary angioedema types I and II

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**Background: We have previously reported that prekallikrein expresses an active site when it is bound to high-molecular-weight kininogen (HK) and can digest HK to produce bradykinin. The reaction is stoichiometric and inhibited by C1 inhibitor (C1-INH) or corn trypsin inhibitor. Addition of heat shock protein 90 leads to conversion of prekallikrein to kallikrein in a zinc-dependent reaction.**

**Objective: Our goal was to determine whether these reactions are demonstrable in plasma and distinguish them from activation through factor XII.**

**Methods: Plasma was incubated in polystyrene plates and assayed for kallikrein formation. C1-INH was removed from factor XII–deficient plasma by means of immunoadsorption.**

**Results: We demonstrate that prekallikrein-HK will activate to kallikrein in phosphate-containing buffers and that the rate is further accelerated on addition of heat shock protein 90. Prolonged incubation of plasma deficient in both factor XII and C1-INH led to conversion of prekallikrein to kallikrein and cleavage of HK, as was seen in plasma from patients with hereditary angioedema but not plasma from healthy subjects.**

**Conclusions: These results indicate that C1-INH stabilizes the prekallikrein-HK complex to prevent HK cleavage either by prekallikrein or by prekallikrein-HK autoactivation to generate kallikrein. In patients with hereditary angioedema, kallikrein and bradykinin formation can occur without invoking factor XII activation, although the kallikrein formed can rapidly activate factor XII if it is surface bound. (J Allergy Clin Immunol 2013;132:470-5.)**

***Key words:*** *Bradykinin, factor XII, prekallikrein, kininogen, C1 inhibitor*

Prekallikrein acquires enzymatic activity on binding to high-molecular-weight kininogen (HK), which results in HK cleavage to release bradykinin.[1] The reaction is stoichiometric; that is, the HK cleavage and bradykinin released are proportional to the prekallikrein input. Firm binding is required rather than the transient enzyme-substrate complex seen with Michaelis-Menten kinetics, and thus a peptide (WIP27) that interferes with binding prevents HK cleavage.[1] Prekallikrein does not possess any demonstrable enzymatic activity unless bound to HK, which is consistent with an active site induced on binding. Under normal circumstances, in a plasma milieu approximately 70% of prekallikrein is bound to HK as a bimolecular complex.[2,3] The reason HK is not cleaved in normal plasma appears to be caused by inhibition of HK cleavage by C1 inhibitor (C1-INH). Corn trypsin inhibitor (CTI) also inhibits cleavage of HK by prekallikrein and is of particular interest because in contrast to C1-INH, CTI does not inhibit kallikrein, thereby distinguishing the active site of prekallikrein (within the complex) from kallikrein.

Heat shock protein 90 (Hsp90) is an endothelial cell–derived factor that binds to the prekallikrein-HK complex, inducing gradual conversion of prekallikrein to kallikrein.[4] This reaction is stoichiometric and requires zinc ion for binding so that the formation of kallikrein is directly proportional to the Hsp90 input. Because this reaction is inhibited by CTI, as well as C1-INH, the active site within the prekallikrein-HK complex appears requisite. Hsp90 has no known proteolytic activity, and therefore a working hypothesis regarding its mechanism is that prekallikrein can autoactivate when simultaneously bound to HK and Hsp90. No conversion of prekallikrein to kallikrein is observed on incubation with Hsp90 in the absence of HK in buffers lacking phosphate ion.

In this article we further characterize these factor XII–independent reactions and demonstrate that they can be observed in plasma, even factor XII–deficient plasma, but only if C1-INH is absent. Thus the C1-INH deficiency seen in patients with hereditary angioedema (HAE) types I or II is a circumstance in which the mechanisms for bradykinin formation discerned herein could be operative. Factor XII is a key initiating protein for bradykinin formation,[5,6] and bradykinin is the cause of angioedema in patients with HAE,[7,8] yet our observations provide alternatives by which attacks of swelling might be initiated.

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*Abbreviations used*
APMSF: (p-Amidinophenyl)methanesulfonyl fluoride
C1-INH: C1 inhibitor
CTI: Corn trypsin inhibitor
HAE: Hereditary angioedema
HK: High-molecular-weight kininogen
Hsp90: Heat shock protein 90

## METHODS

### Collection of plasma

Plasma was collected from patients with HAE and healthy control subjects by mixing 1 mL of 3.2% sodium citrate with 9 mL of blood (total of 10 mL) and centrifuged at 1200*g* for 10 minutes. The protocol was approved by the Danish Ethics Committee and the Danish Data Protection Agency. The collected plasma was stored at −80°C.

### SDS-PAGE and Western blotting

Samples were collected in SDS sample buffer containing 5% 2-mercaptoethanol. SDS-PAGE was performed by using the buffer system of Laemmli.[9] Four percent to 20% gradient gels were used for separation of proteins. After electrophoresis, the separated proteins from gels were transferred to nitrocellulose membranes overnight. The membranes were then incubated with blocking buffer (1% BSA in PBS) for 1 hour and probed with mAbs for an additional hour. Bound probes were visualized by incubating the membranes with alkaline phosphatase–conjugated secondary antibodies, followed by color development in 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

### Prekallikrein activation assay

Prekallikrein activation assays were performed in assay buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 4 mmol/L KCl, 11 mmol/L D-glucose, and 1 mg/mL RIA grade BSA) with a kallikrein-specific substrate (0.6 mmol/L S2302) in 96-well disposable polystyrene microtiter plates (Dynatech Laboratories, Chantilly, Va) pretreated with 1% polyethylene glycol (Aquacide III; Calbiochem, La Jolla, Calif). Just before the assay, all the proteins were treated with 2.0 mmol/L (p-Amidinophenyl)methanesulfonyl fluoride (APMSF) for 90 minutes at pH 5.5, after which they were diluted 1:100 with assay buffer and incubated for 30 minutes to allow for the decomposition of any unreacted APMSF at the neutral pH. Assay buffer was also pretreated with 0.4 μmol/L APMSF to inactivate any serene protease activity present in the RIA grade BSA. HK and prekallikrein were incubated in the assay buffer in the presence of S2302, and the kallikrein activity was determined based on color development. Absorbance (OD of 405 nm) was monitored at room temperature on a Molecular Devices (Sunnyvale, Calif) THERMOmax microplate reader.

### Immunoadsorption of C1-INH

For immunoadsorption of C1-INH, factor XII–deficient plasma (George-King Biomedical, Overland Park, Kan) was passed over a C1-INH antibody affinity column, and the flow-through was collected. Plasma passed through a Sepharose column without the antibody was used as a control.

## RESULTS

### Activation of plasma bradykinin-forming pathway in plasma by prolonged incubation

Plasma from patients with HAE and normal control plasma were incubated in 96-well polystyrene plates precoated with 1% polyethylene glycol. This was chosen because polystyrene is not known to be an activator of factor XII and because polyethylene glycol prevents binding of proteins to surfaces in a nonspecific fashion. Samples were collected at time 0, 1 hour, 2 hours, and 4 hours. SDS-PAGE and Western blotting with antibody to the light chain of HK revealed no activation of HK in normal plasma (Fig 1, *A*, upper left panel). On incubation of plasma from patients with HAE (Fig 1, *A*, upper right panel), there was progressive digestion of HK with conversion to heavy and light chains. This activation of HK could be due to HK digestion by either prekallikrein or kallikrein. The latter circumstance could be due to factor XII activation, the presence of Hsp90 in the plasma, or autoactivation of prekallikrein to kallikrein. There was a lesser baseline HK level in this particular plasma from patients with HAE, and some conversion to the heavy and light chain was seen, even at time 0; plasma from additional patients with HAE behaved similarly, although the baseline HK level varied from normal to low.

When the same samples were assayed for the presence of kallikrein by using SDS-PAGE, there was none evident in the control plasma (Fig 1, *A*, bottom left panel), whereas the plasma from patients with HAE had identifiable kallikrein at 4 hours but not at the earlier time points. Thus small amounts of kallikrein could account for the HK cleavage seen in plasma from patients with HAE at 1, 2, and 4 hours, so that the results do not allow distinction of prekallikrein from kallikrein at the earlier time points. When factor XII was assessed by using SDS-PAGE, no conversion to factor XIIa or factor XIIf could be seen in either plasma (data not shown). Thus the possibility remains that the kallikrein seen at 4 hours is not due to factor XII activation unless the amount of activated factor XII required is less than could be demonstrated by using this method.

We next assayed the same samples for the presence of kallikrein using Pro-Phe-Arg-p-nitroanilide (which is not digested by the prekallikrein-HK complex, even when prekallikrein cleaves all the HK). As shown in Fig 1, *B*, there is detectable kallikrein formed even at very early time points, yet it is insufficient in quantity to be seen on an SDS gel (Fig 1, *A*, lower panel). Normal plasma yields no detectable kallikrein by using this method.

To determine whether addition of exogenous Hsp90 is capable of activating the prekallikrein-HK complex to yield kallikrein in normal plasma, we added Hsp90 and incubated the samples using the same conditions and analysis used in Fig 1, *A*. There was prompt conversion of prekallikrein to kallikrein in the presence of Hsp90 (Fig 1, *C*), although not in its absence. When analyzed by using SDS-PAGE, there was progressive HK cleavage with time, but kallikrein formation was clearly visible only at 4 hours (Fig 1, *D*). Thus we observed the effect of Hsp90 to generate kallikrein in normal plasma, even in the presence of C1-INH.

### Activation of prekallikrein and HK using purified proteins

To determine the possibility of bradykinin formation in the absence of factor XII and C1-INH, we incubated purified prekallikrein and HK in binding buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 4 mmol/L KCl, 11 mmol/L D-glucose, and 1 mg/mL RIA grade BSA, pH 7.4), and samples were analyzed. There was gradual cleavage of HK, and by 4 hours, digestion was complete (Fig 2, *A*). When the same samples were analyzed for prekallikrein, there was no conversion of prekallikrein to kallikrein (Fig 2, *B*). This defines HK cleavage by prekallikrein, as previously described.[1] The addition of