

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;■■■:■■■-■■■.)

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 1200g 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 $\mu\text{mol/L}$ APMSF to inactivate any serine 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.

Immunoabsorption of C1-INH

For immunoabsorption 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

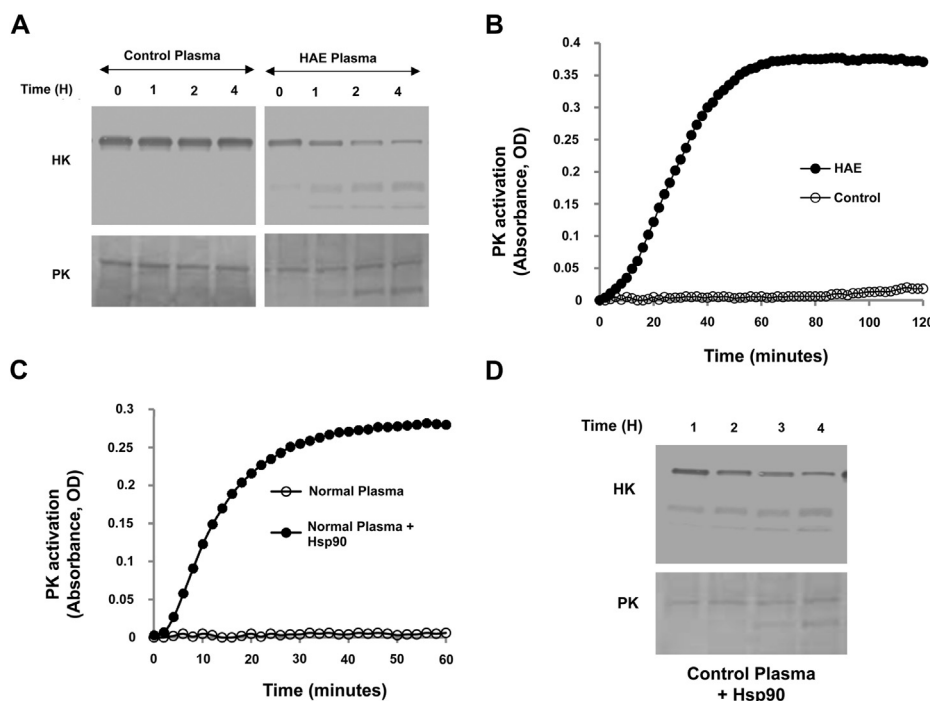


FIG 1. Activation of the plasma bradykinin-forming pathway by prolonged incubation. Plasma was incubated in polyethylene glycol-coated 96-well polystyrene plates at 37°C. Samples were withdrawn at the indicated time points, and the reaction was stopped by the addition of SDS sample buffer. **A**, Western blot of samples from control plasma (*left*) and plasma from patients with HAE (*right*) using antibody to the light chain of HK (*upper panel*) or antibody to prekallikrein (*lower panel*). Progressive HK cleavage with time is evident in plasma from patients with HAE but not in normal plasma. Formation of kallikrein is also evident in plasma from patients with HAE and not in control plasma. **B**, Assay of kallikrein activity in plasma from patients with HAE and control plasma using a specific synthetic substrate (Pro-Phe-Arg-p-nitroanilide). **C**, Comparison of kallikrein activity in normal plasma and in the presence of Hsp90. **D**, Western blot demonstrating addition of Hsp90 to normal plasma reveals HK cleavage and kallikrein formation by the 4-hour time point. PK, Prekallikrein.

CTI to the prekallikrein-HK mixture completely prevents HK cleavage (Fig 2, C). CTI has no effect on plasma kallikrein but is known to inhibit all forms of activated factor XII.¹⁰ Because there was no factor XII in this reaction, we confirmed that the active site in prekallikrein is susceptible to inhibition by CTI, whereas kallikrein is not.

We next repeated the experiment on addition of Hsp90 to the reaction mixture. As shown in Fig 3, A, HK cleavage is very rapid in the presence of Hsp90; virtually all of it is digested by 30 minutes, whereas small amounts of kallikrein are evident from 30 minutes to 4 hours (Fig 3, B). When CTI is added, there is no conversion of prekallikrein to kallikrein, and HK is not digested (Fig 3, C). This demonstrates the marked potency of kallikrein versus prekallikrein for HK cleavage and suggests that the site in prekallikrein that digests HK will catalyze prekallikrein autoactivation in the presence of Hsp90. The inhibition of both reactions by CTI indicates that the same active site might be responsible for both enzymatic reactions.

Earlier reports suggested marked augmentation of factor XII-dependent activation of the bradykinin-forming cascade in phosphate buffer.¹¹ Because all the buffers we used were free of phosphate and because plasma is buffered by phosphate ion, we checked the effect of phosphate ion in the factor XII-independent system (ie, purified proteins). When prekallikrein and HK were incubated in phosphate buffer in the absence of

Hsp90, the HK is cleaved at least as rapidly (or faster) in phosphate buffer (Fig 4, A), and prekallikrein is converted to kallikrein (Fig 4, B). Thus in phosphate buffer it is clear that prekallikrein autodigests and autoactivates to produce kallikrein, so that the increased rate of HK digestion is due to kallikrein. This reaction is still inhibited by CTI (Fig 4, C), so that conversion of prekallikrein to kallikrein is prevented (ie, CTI inhibits the site in prekallikrein responsible for autoactivation). The antibody used to visualize HK is specific for the light chain, and it has been shown that an initial 56K light chain is converted to a 45K light chain over time.^{12,13} The Hsp90 experiment clearly depicts a mixture of these 2 light chain forms (Fig 3, A), whereas the 56K light chain is rapidly converted to the 42K light chain in phosphate buffer (Fig 4, A). Thus in phosphate buffer we cannot demonstrate prekallikrein cleavage of HK because the prekallikrein autoactivates to kallikrein. This might limit the ability to definitively demonstrate prekallikrein cleavage of HK in plasma.

Role of zinc versus phosphate in prekallikrein activation

We have shown that Hsp90-dependent conversion of prekallikrein to kallikrein within the prekallikrein-HK complex is dependent on zinc ion, and therefore all buffers used to demonstrate this reaction contain 50 μmol/L zinc. The ability of

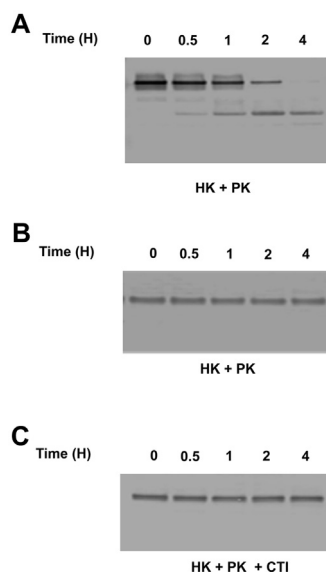


FIG 2. Activation of HK and prekallikrein using purified proteins. HK (50 $\mu\text{g/mL}$) and prekallikrein (50 $\mu\text{g/mL}$) were incubated at room temperature, and samples were collected at the indicated time points. **A**, Western blot with antibody to the light chain of HK, demonstrating cleavage of HK by prekallikrein. **B**, Western blot with antibody to prekallikrein, demonstrating that prekallikrein is not converted to kallikrein on incubation with HK. **C**, Western blot with antibody to prekallikrein. PK, Prekallikrein.

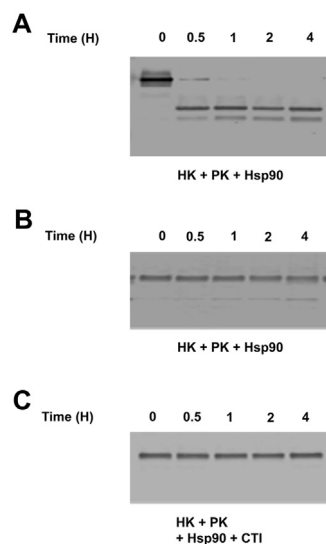


FIG 3. Activation of HK and prekallikrein in the presence of Hsp90. HK (50 $\mu\text{g/mL}$) and prekallikrein (50 $\mu\text{g/mL}$) were incubated in the presence of Hsp90 (50 $\mu\text{g/mL}$), and samples were collected at the indicated time points. **A**, Western blot with antibody to the light chain of HK, demonstrating cleavage of HK by prekallikrein, followed by more rapid cleavage of HK on addition of Hsp90. **B**, Western blot with antibody to prekallikrein, demonstrating progressive formation of kallikrein on addition of either Hsp90. **C**, Western blot with antibody to prekallikrein. Complete inhibition of prekallikrein conversion to kallikrein is obtained on addition of CTI. PK, Prekallikrein.

prekallikrein to activate HK without any kallikrein being present does not require zinc ion. Because the presence of phosphate ion resembles the complex interactions observed when Hsp90 is present, we next compared activation in the presence of only zinc,

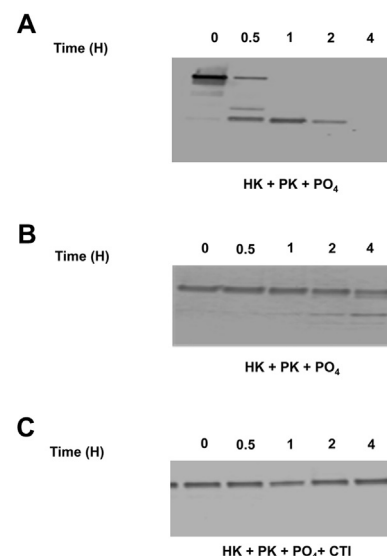


FIG 4. Activation of HK and prekallikrein in phosphate buffer. HK (50 $\mu\text{g/mL}$) and prekallikrein (50 $\mu\text{g/mL}$) were incubated in phosphate (20 mmol/L)-containing buffer, and samples were collected at the indicated time points. **A**, Western blot with antibody to the light chain of HK, demonstrating rapid cleavage of HK if the buffer contains phosphate. **B**, Western blot with antibody to prekallikrein, demonstrating progressive formation of kallikrein on addition of phosphate. **C**, Complete inhibition of prekallikrein conversion to kallikrein is obtained on addition of CTI. PK, Prekallikrein.

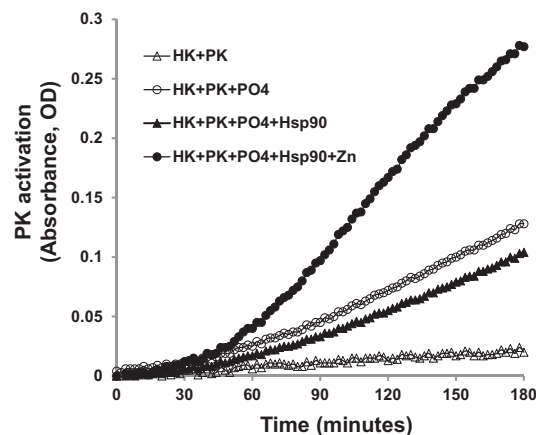


FIG 5. Prekallikrein activation by Hsp90 or phosphate: role of zinc versus phosphate. HK (50 $\mu\text{g/mL}$) and prekallikrein (50 $\mu\text{g/mL}$) were incubated in phosphate (20 mmol/L)-containing buffer in the presence or absence of Hsp90, and kallikrein activity was measured by using a synthetic substrate. Conversion of prekallikrein to kallikrein requires binding to HK plus phosphate buffer. PK, Prekallikrein.

only phosphate, or a combination of both. As shown in Fig 5, when prekallikrein and HK were incubated in the absence of zinc or phosphate, there was no evidence of kallikrein activity. Note, however, that the HK is digested to release bradykinin (Figs 2-4). The addition of phosphate or phosphate plus Hsp90 yielded conversion of prekallikrein to kallikrein, which were not distinguishable. When zinc was added in addition to phosphate and Hsp90, the Hsp90 effect was augmented beyond the phosphate effect, and more rapid conversion of prekallikrein to kallikrein was observed (Fig 5).

Demonstration of factor XII-independent prekallikrein activation in plasma when C1-INH is absent

Although interpretation of our observations appear clear when using purified proteins, any conversion of prekallikrein to kallikrein caused by Hsp90 or to the phosphate ion present is limited by the fact that even minute amounts of activated factor XII can account for the observation. Furthermore, any kallikrein formed can activate factor XII.^{14,15} Nevertheless, in the absence of addition of a factor XII activator, normal plasma does not appear to spontaneously activate on prolonged incubation because of the C1-INH present (Figs 1 and 2). Therefore we obtained factor XII-deficient plasma (factor XII level, <1.0%) and removed the C1-INH using immunoabsorption. We then incubated the plasma under the conditions used for Figs 1 and 2 and measured conversion of prekallikrein to kallikrein. As can be seen in Fig 6, the factor XII-deficient plasma with normal C1-INH levels does not activate (as expected); however, once C1-INH is removed, prekallikrein is converted to kallikrein, demonstrating autoactivation of the prekallikrein-HK complex as seen with purified proteins in a buffer containing phosphate ion.

DISCUSSION

The results with purified proteins clearly demonstrate that prekallikrein expresses an active site when bound to HK. This site is inhibited by C1-INH, as well as CTI. The prekallikrein-HK complex, if incubated in a non-phosphate-containing buffer, will lead to stoichiometric HK cleavage and liberation of bradykinin proportionately. When Hsp90 is added to the prekallikrein-HK complex, conversion of the prekallikrein to kallikrein will proceed but requires zinc ion. It has been reported that endothelial cell-derived Hsp90 does not convert prekallikrein to kallikrein unless it is bound to HK.⁴ Because neither Hsp90 nor HK are known to be proteolytic enzymes, we conclude that prekallikrein will autodigest and autoactivate when bound to both. Because autoactivation seemed to proceed in phosphate buffer without Hsp90, we sought to define the role, if any, of HK binding. Using a functional assay, namely cleavage of Pro-Phe-Arg-p-nitroanilide, we demonstrate that conversion of prekallikrein to kallikrein in a phosphate buffer only occurs if it is bound to HK (Fig 3). Thus prekallikrein, as synthesized from the mRNA, does not have a demonstrably active site but acquires one when it is bound to HK (ie, the active site is induced on binding).

Binding of prekallikrein to both Hsp90 and HK leads to prekallikrein autodigestion and autoactivation to kallikrein, thus providing a mechanism by which factor XII could be activated secondarily,¹⁶ with prekallikrein activation as the initiating event. The Hsp90-prekallikrein-HK reaction is inhibited by C1-INH and therefore would be relatively uncontrolled in patients with HAE. A surprising finding is that prekallikrein autoactivation can proceed in phosphate buffer in the absence of Hsp90 and that zinc is not needed. This emphasizes that interaction of Hsp90 with the prekallikrein-HK complex specifically requires zinc. Thus in a non-phosphate-containing buffer, prekallikrein slowly digests HK, whereas in phosphate buffer, a competing reaction involves prekallikrein-HK molecules digesting each other to produce kallikrein, which then accelerates the HK cleavage so that prekallikrein activation of HK is not clearly discerned. Yet CTI, which does not inhibit kallikrein, stops prekallikrein-HK

1. Prekallikrein-HK	(-) phosphate	→ HK cleavage
2. Prekallikrein-HK	(+) phosphate	→ HK cleavage, Prekallikrein autoactivation
3. Prekallikrein-HK	(+) Hsp90, (+) Zn	→ HK cleavage, Prekallikrein activation
4. Prekallikrein-HK	(+) phosphate (+) Hsp90	→ HK cleavage, Prekallikrein autoactivation
5. Prekallikrein-HK	(+) phosphate, (+) Zn (+) Hsp90	→ Augmented Prekallikrein autoactivation

FIG 6. A summary of reactions in the absence of factor XII. Digestion of HK by prekallikrein (reaction 1) and conditions for conversion of prekallikrein to kallikrein, as well as HK cleavage (reactions 2-5) to produce bradykinin. Specific requirements are shown along the arrow. PK, Prekallikrein.

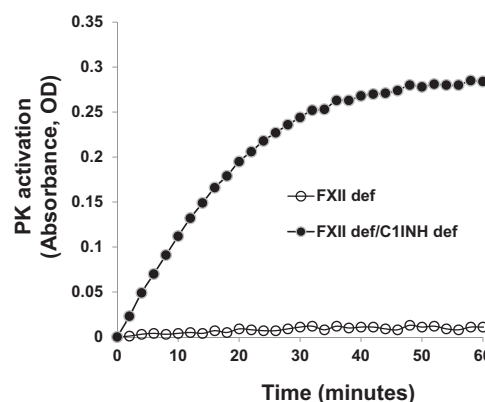


FIG 7. Factor XII-independent prekallikrein activation in plasma. Factor XII-deficient plasma was passed over a C1-INH antibody affinity column to remove C1-INH. Plasma were incubated in polyethylene glycol-coated 96-well polystyrene plates at 37°C, and kallikrein activity was measured with a synthetic substrate. FXII, Factor XII; PK, prekallikrein.

autoactivation completely and therefore interacts with the active site in prekallikrein. The addition of zinc to the Hsp90-prekallikrein-HK complex in phosphate buffer adds the effect of Hsp90, and the rate of prekallikrein autoactivation is further augmented (Fig 5). A summary of the reactions is given in Fig 6.

Prolonged incubation of plasma from patients with HAE versus normal control plasma leads to complete HK cleavage in plasma from patients with HAE and conversion of prekallikrein to kallikrein only at later time points. This effect is dependent on C1-INH and is not observed in normal plasma. The HK cleavage can be due to the active site in prekallikrein, with prekallikrein digestion of HK initially, followed by kallikrein digestion of HK as prekallikrein autoactivation proceeds, given the phosphate present in plasma. However, we could not exclude a role for trace amounts of activated factor XII in the conversion of prekallikrein to kallikrein. However, our observation of kallikrein formation in combined factor XII- and C1-INH-deficient plasma reproduces the observations seen with purified proteins (Fig 7). The acquisition of this active site in prekallikrein as a result of binding to HK is a new concept for the initiation of bradykinin formation and an alternative to factor XII autoactivation⁵ as an initiating mechanism and might be particularly relevant in patients with HAE types I and II caused by C1-INH deficiency.¹⁷

Because factor XII does not contain an active site,¹⁸ as does the prekallikrein-HK complex, the origin of the putative trace quantities of factor XIIa needed to initiate factor XII autoactivation have never been defined, but the theoretical requirement is at least 1000 times less than our ability to measure factor XIIa levels in plasma.¹⁸ One source of this factor XIIa could be factor XII activation through the prekallikrein-HK complex. Thus considerations of the initiation of attacks of swelling in patients with HAE can include a role not only for activation of factor XII but also for activation of the prekallikrein-HK complex. Endothelial cell activation to secrete Hsp90 (eg, infection and other stressful circumstances) is but one of many theoretical possibilities.

Clinical implications: Plasma of patients with HAE is intrinsically unstable because C1-INH is absent. Factor XII-independent activation of the prekallikrein-HK complex can be an important mechanism for initiating bradykinin formation.

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