Cytokine and estrogen stimulation of endothelial cells augments activation of the prekallikrein-high molecular weight kininogen complex: Implications for hereditary angioedema

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Background: When the prekallikrein-high molecular weight kininogen complex is bound to endothelial cells, prekallikrein is stoichiometrically converted to kallikrein because of release of heat shock protein-90 (Hsp90). Although bradykinin formation is typically initiated by factor XII autoactivation, it is also possible to activate factor XII either by kallikrein, thus formed, or by plasmin.
Objective: Because attacks of hereditary angioedema can be related to infection and/or exposure to estrogen, we questioned whether estrogen or cytokine stimulation of endothelial cells could augment release of Hsp90 and prekallikrein activation. We also tested release of profibrinolytic enzymes, urokinase, and tissue plasminogen activator (TPA) as a source for plasmin formation.
Methods: Cells were stimulated with agonists, and secretion of Hsp90, urokinase, and TPA was measured in the culture supernatants by ELISA. Activation of the prekallikrein-HK complex was measured by using pro-phe-arg-p-nitroanilide reflecting kallikrein formation.
Results: Hsp90 release was stimulated with optimal doses of estradiol, IL-1, and TNF-α (10 ng/mL) from 15 minutes to 120 minutes. TPA release was not augmented by any of the agonists tested but urokinase was released by IL-1, TNF-α, and thrombin (positive control), but not estrogen. Augmented activation of the prekallikrein-HK complex to generate kallikrein was seen with each agonist that releases Hsp90. Addition of 0.1% factor XII relative to prekallikrein-HK leads to rapid formation of kallikrein; factor XII alone does not autoactivate.
Conclusions: IL-1, TNF-α, and estrogen stimulate release of Hsp90 and augment activation of the prekallikrein-HK complex to generate kallikrein and bradykinin. IL-1 and TNF-α stimulate release of urokinase, which can convert plasminogen to plasmin and represents a possible source for plasmin generation in all types of hereditary angioedema, but particularly hereditary angioedema with normal C1 inhibitor with a factor XII mutation. Both kallikrein and plasmin activate factor XII; kallikrein is 20 times more potent on a molar basis. (J Allergy Clin Immunol 2017;140:170-6.)

Key words: *Hereditary angioedema, C1 inhibitor, bradykinin, heat shock protein 90*

The timing of attacks of angioedema in patients with the hereditary forms (types I and II; C1 inhibitor deficiency) or type III (hereditary angioedema with normal C1 inhibitor [HAE-N]) is, in general, unpredictable. However, a number of "triggers" that seem to associate with the onset of angioedema include physical trauma, infection, stress including emotional stress, and use of estrogen. How these actually lead to an augmented surge in bradykinin production is not clear, but the association is assumed, because the final step in angioedema formation is the interaction of bradykinin with venular B2 receptors to produce vasodilation and an increase in permeability. It is known that estrogen raises blood levels of factor XII,[1-3] which can enhance bradykinin formation once initiation of an event occurs. Trauma, for example, may expose plasma or interstitial fluid to vessel wall constituents such as collagen and sulfated proteoglycans. Although type I collagen has been reported to function as a "surface" to initiate activation of factor XII,[4] the role of collagen itself, when highly purified, has been controversial[5,6]; highly sulfated proteoglycans (heparin, heparan sulfate, or chondroitin sulfates), however, clearly bind factor XII to initiate factor XII autoactivation[7] as a first step to bradykinin formation.[8-10]

We have approached this question using a model in which the constituents of the bradykinin-forming cascade are bound to the cell surface and the endothelial cells stimulated in such a way as to approximate what might occur during an initiating event. We have chosen conditions of cytokine stimulation with IL-1 and TNF-α, estrogen, and bradykinin itself as cell stimuli and focus on release of heat shock protein 90 (Hsp90), a transport protein that can be expressed at the cell surface and secreted into the milieu. Hsp90 acts on the complex of prekallikrein-high molecular weight kininogen (prekallikrein-HK) to generate the enzyme kallikrein.[11] Kallikrein, in turn, activates factor XII and cleaves HK to release bradykinin. Because plasmin is an alternative enzyme capable of activating factor XII,[12] we also monitored secretion of profibrinolytic enzymes urokinase and TPA.

METHODS

Materials

Human plasma proteins, HK, prekallikrein, and factor XII, were purchased from Enzyme Research Laboratories (South Bend, Ind).

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Abbreviations used
APMSF: (4-Amidinophenyl)-methanesulfonyl fluoride
HAE: Hereditary angioedema
HK: High molecular weight kininogen
PAI-2: Plasminogen activator inhibitor-2
TPA: Tissue plasminogen activator

HK, prekallikrein, and factor XII were treated with 0.1 mmol/L (4-amidinophenyl)-methanesulfonyl fluoride (APMSF; Boehringer Mannheim, Indianapolis, Ind) before storage in 4 mmol/L sodium acetate buffer containing 0.15 mol/L NaCl (pH 5.5) at −80°C. The synthetic substrate H-D-prolyl-D-phenylalanyl-D-arginine p-nitrophenylester (S2302) was purchased from Kabi Pharmacia, Inc (Franklin, Ohio). Purified Hsp90 was purchased from R&D Systems (Minneapolis, Minn). Protease inhibitors and other reagents were obtained from Sigma (St Louis, Mo).

Collection of citrated plasma. Citrated plasma used in this study was separated by centrifugation of freshly collected blood at 2000 rpm for 10 minutes at 4°C. Samples were immediately aliquoted and stored at −80°C.

Endothelial cell culture. Human umbilical vein endothelial cells, used in all cell-dependent experiments, were isolated as described earlier[13] and cultured in gelatin-coated dishes in endothelial growth medium (EGM Bullet kit, Clonetics, San Diego, Calif). For cell stimulation experiments, cells were grown in 24-well plates in 0.5 mL culture medium. For prekallikrien and factor XII activation assays, the cells were subcultured into 96-well plates in 0.2 mL of culture medium. The cells were identified as endothelial cells by their distinct cobblestone morphology and interaction with antiserum to Von Willebrand's factor.[14] All the cells were used at the third or fourth cell passage.

Direct binding of HK and prekallikrein to Hsp90. Purified Hsp90 was coated onto microtiter plates by incubating in 0.1 mol/L Na_2CO_3, pH 9.6, overnight at 4°C. After blocking with 1% BSA in PBS, various concentrations of HK and prekallikrein in Hepes buffered saline containing 0.5% BSA and 50 μM zinc chloride were incubated up to 2 hours at 37°C. The relative binding of each protein was determined using specific antibodies followed by alkaline phosphatase–conjugated secondary antibodies and color development using phosphatase-specific substrate.

SDS-polyacrylamide gel electrophoresis and Western blot. Samples were collected in SDS sample buffer containing 5% 2-mercaptoethanol. SDS-polyacrylamide gel electrophoresis was performed using the buffer system of Laemmli.[15] Gradient gels (4% to 20%) 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 1 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/nitro blue tetrazolium.

Prekallikrein activation assay

PK activation assays were performed in "assay buffer" (10 mM Hepes, 137 mM NaCl, 4 mM KCl, 11 mM D-glucose, 1 mg/mL RIA grade BSA), with a kallikrein-specific substrate (0.6 mM 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 mM 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 μM APMSF to inactivate any serene protease activity present in the RIA grade BSA. HK and PK were incubated in the assay buffer in the presence of S2302 and the kallikrein activity was determined by the color development. The absorbance (OD at 405 nm) was monitored at room temperature on a Molecular Devices (Sunnyvale, Calif) THERMOmax microplate reader.

ELISA to measure levels of Hsp90, urokinase, and tissue-type plasminogen activator. ELISA kits for the measurement of Hsp90 were from Enzo Life Sciences, Inc (Farmingdale, NY). Assay kits for urokinase and TPA were obtained from Cloud-Clone Corp (Houston, Tex). All assays were performed according to the manufacturer's recommendations.

Statistical analysis

Statistical analysis was performed using paired *t* test. Values obtained from each experimental condition (time course and dose-response) were compared with corresponding control values. A *P* value of less than .05 was considered significant.

RESULTS

Binding of HK and prekallikrein to Hsp90

The interaction of HK-prekallikrein with Hsp90 is stoichiometric and also dependent on zinc ion[11]; however, it is not clear whether Hsp90 binds to HK or prekallikrein. We therefore examined the ability of microtiter plates coated with Hsp90 (5 μg/mL) to bind either HK or prekallikrein. As can be seen in Fig 1, Hsp90 binds primarily to HK (saturated at 5 μg/mL), thereby forming a trimolecular complex with HK-prekallikrein although it interacts with prekallikrein as well. There are no interactions in the absence of zinc.

Hsp90 release from activated endothelial cells

Human umbilical vein endothelial cells were incubated with increasing doses of IL-1, TNF-α, and estradiol and the release of Hsp90 was measured by ELISA using monospecific antibody. An optimal dose was, coincidentally, about 10 ng/mL in each case (Fig 2, *A*), which is within the range of previous reports.[16,17] A time course of cell activation is shown in Fig 2, *B*. Compared with the control, there was increased release at 15, 30, 60, and 120 minutes using estradiol with a clear dose-response, and similar increases are seen at 15, 30, and 60 minutes using IL-1. For the latter, the peak level decreased at 120 minutes. The result with TNF-α was not as robust, but was increased at the 60- and 120-minute time points. We know that estrogen and infection can be triggers of angioedema in types I and II HAE and most cases of type III (HAE with normal C1 inhibitor) are strikingly estrogen dependent.[18-20] This provides one mechanism by which bradykinin generation could be linked to these substances.

Kallikrein formation on IL-1-β–, TNF-α–, and estrogen-stimulated endothelial cells

Based on the above findings, it was evident that endothelial cell stimulation can release Hsp90. Next, we questioned whether it can be connected to the formation of bradykinin or as a surrogate, the conversion of prekallikrein to kallikrein. The results are shown in Fig 3 where we compared prekallikrein alone, HK-prekallikrein alone, and HK-prekallikrein stimulated with IL-1β, TNF-α, or estrogen. Stimulation of endothelial cells with IL-1-β, TNF-α, and estrogen does not augment kallikrein formation when prekallikrein alone is bound to the cell. In contrast, HK-prekallikrein does slowly activate but there is augmentation of HK-prekallikrein conversion to kallikrein when the cells were stimulated by IL-1-β, estrogen, or TNF-α and all these are strikingly similar at the 10 ng/mL concentrations