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Pleiotropic effects of a vibrio extracellular protease on the activation of contact system

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ABSTRACT

Many proteases secreted by pathogenic bacteria can affect seriously on hemostatic system. We have reported that an extracellular zinc metalloprotease (named vEP-45) from *Vibrio vulnificus* ATCC29307 activates prothrombin to active thrombin, leading the formation of fibrin clot. In this study, the effects of vEP-45 on the intrinsic pathway of coagulation and the kallikrein/kinin system were examined. The protease could activate proteolytically clotting factor zymogens, including FXII, FXI, FX, and prothrombin, to their functional enzymes *in vitro* and plasma milieu. In addition, it could cleave plasma prekallikrein (PPK) to form an active kallikrein as well as actively digest high-molecular weight kininogen (HK), probably producing bradykinin. In fact, vEP-45 could induce a vascular permeability in a dose-dependent manner *in vivo*. Taken together, the results demonstrate that vEP-45 can activate plasma contact system by cleaving key zymogen molecules, participating in the intrinsic pathway of coagulation and the kallikrein/kinin system.

1. Introduction

Plasma contact system, integral part of innate immunity, is composed of the intrinsic pathway of coagulation and the kallikrein/kinin system [1–3]. Four major factors control this system in a cascade manner [2]. These include three serine protease zymogens [factor XII (FXII), factor XI (FXI) and plasma prekallikrein (PPK)] and a non-enzymatic protein, high-molecular weight kininogen (HK). Of these factors, FXII acts as an initiator to activate the contact system cascade. At the initial stage of activation, FXII zymogen is auto-activated to active FXII (FXIIa) through a conformational change when it contacts to negatively charged surfaces such as kaolin, heparin, and dextran sulfate [1], when the endothelium that lines the blood vessel is changed to pro-coagulant state [4], and/or when the platelets are stimulated to release polyphosphates (also called Poly P) [2]. The activated FXIIa cleaves then PPK to produce active kallikrein (Kall), which in turn digest HK to release proinflammatory nonapeptide bradykinin (BK), causing the expansion of blood vessels and also inducing vascular permeability [5–7]. At the same time, FXIIa also triggers the activation of the intrinsic pathway of coagulation by converting FXI to active FXI (FXIa) that activates proteolytically FX zymogen to active FX (FXa). The FXa generated cleaves prothrombin to make thrombin that cleaves fibrinogen, leading to the formation of cross-linked fibrin [3]. The proteolytic activities of FXIIa, FXIa, and Kall are controlled by an endogenous typical serpin, C1 esterase inhibitor (also known as C1-INH), to be inhibited [3,8].

Bacteria secrets a variety of proteases involved in activating the kallikrein/kinin system and the intrinsic pathway of coagulation [3]. In fact, many bacterial proteases act as activators for plasma zymogens, which are participating in the activation of the contact system [3,8,9]. In previous studies, we have reported that an opportunistic pathogenic marine bacterium *Vibrio vulnificus* (*V. vulnificus*) ATCC29307 secretes a zinc metalloprotease (named vEP-45) that interferes with blood homeostasis through prothrombin activation and fibrinolysis [10,11]. vEP-45 is a broad-specificity protease and cleaves actively various blood clotting-associated plasma proteins such as prothrombin and fibrinogen [10]. Furthermore, a peptide derived from vEP-45-cleaved prothrombin shows an actual thrombin activity capable of producing functional

Abbreviations: 1,10-PT, 1,10-phenanthroline; AFC, 7-amido-4-trifluormethylcoumarin; BK, bradykinin; FX, factor X; FXI, factor XI; FXII, factor XII; HK, high-molecular weight kininogen; Kall, kallikrein; PBS, phosphate buffered saline; PPK, plasma prekallikrein; *p*NA, *para*-nitroaniline; PT, prothrombin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; serpin, serine proteinase inhibitor; TH, thrombin; vEP, vibrio extracellular protease.

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fibrin monomers, which are spontaneously polymerized and cross-linked in the presence of FXIIIa [10,11].

These previous results led us to examine what influence vEP-45 might have on the activation of contact system *in vitro* and plasma milieu. We describe here the pleiotropic effects of vEP-45 protease on the activation of kallikrein/kinin system and the intrinsic pathway of coagulation *in vitro* and under plasma milieu, together with its involvement in the induction of vascular permeability in guinea pig system.

2. Materials and methods

2.1. Materials

Various human zymogens and their active enzymes, including FXII, FXIIa, FXI, FXIa, PPK, Kall, and HK were purchased from Enzyme Research Laboratories (IN, USA). Protein size markers were from Fermentas (Darmstadt, Germany). Bradford protein assay kit, 1,10-PT, Evans blue dye, and SDS were obtained from Sigma (St. Louis, MO, USA). Diethyl ether was from Junsei (Tokyo, Japan). Synthetic chromogenic substrates, including H-D-Pro-Phe-Arg-*p*NA (S-2302) and N-α-Z-D-Arg-Gly-Arg-*p*NA (S-2765), were purchased from Chromogenix (Milan, Italy). Boc-Val-Pro-Arg-*p*NA was from Seikagaku (Tokyo, Japan). A fluorogenic peptide substrate (H-D-Val-Leu-Arg-AFC) was from Calbiochem (Darmstadt, Germany).

2.2. Expression and purification of vEP-45 protease and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Escherichia coli (*E. coli*) DH5α cells were cultured in Luria–Bertani (LB) medium as described elsewhere. vEP-45 protease was expressed and purified from *E. coli* DH5α cells harboring a recombinant plasmid pvEP-45 as described previously [10]. SDS–PAGE was performed according to the method of Laemmli [12]. Typically, protein samples were mixed with an equal volume of 2× SDS–PAGE sample buffer, heated at 100 °C for 3 min, and then subjected to electrophoresis on 12% polyacrylamide gel. After electrophoresis, protein bands were visualized by staining the gel with 0.25% Coomassie brilliant blue.

2.3. vEP-45-mediated cleavage of FXII, FXI, PPK, and HK

Reaction mixture consisted of 10 μg each of proteins to be digested (FXII, FXI, PPK, or HK) and 0.2 μg of vEP-45 in a reaction buffer (50 mM Tris–HCl, pH 7.5, 0.9% NaCl, and 0.1 mg/ml BSA) was incubated for 1 or 5 min at 37 °C. Thereafter, the reaction was terminated by the addition of 1 mM of 1,10-PT and the resulting products were electrophoresed on 12% SDS–polyacrylamide gel, followed by staining with 0.25% Coomassie brilliant blue to visualize.

2.4. vEP-45-induced activation of FXII, FXI, FX, and PPK

Reaction mixture consisted of 5 μg each of zymogens (FXII, FXI, FX, or PPK) and 0.2 μg of vEP-45 in the same reaction buffer described in Section 2.3 was incubated for 1 or 5 min at 37 °C and the reaction was terminated by the addition of 1 mM of 1,10-PT. Thereafter, the activated enzyme activities were observed as follows: FXIIa and the FXIa activities were examined with the chromogenic substrate S-2302, in which the increases in absorbance at 405 nm were monitored every 30 s for 10 min at 37 °C in a 96-well plate reader (Molecular Devices). Kall activity was assayed with 0.4 mM of H-D-Val-Leu-Arg-AFC by measuring λ_{em} = 505 nm and λ_{ex} = 400 nm every 30 s at 37 °C for 10 min in a micro spectrofluorometer (Molecular Devices), from which the activity resulted was expressed as the relative fluorescence unit (RFU).

2.5. vEP-45-induced activation of the components of contact system in plasma milieu

Human plasma was prepared as described previously [13] and diluted with phosphate buffered saline (PBS) to a final concentration of 10%. To examine the activation of contact system components, 90 μl of 10% plasma and 10 μl of vEP-45 (1 μg) were mixed in the presence or absence of 1 mM of 1,10-PT and then 0.4 mM each of various synthetic peptide substrates (S-2302 for FXIIa and FXIa, H-D-Val-Leu-Arg-AFC for kallikrein, S-2765 for FXa, and Val-Pro-Arg-*p*NA for thrombin) was added. The activity produced was then monitored every 2 min for 30 min at 37 °C as described in Section 2.4.

2.6. vEP-45-induced vascular permeability in guinea pig

Evans blue dye was dissolved in PBS to a final concentration of 5% and filtered through a sterilized membrane (0.2 μm in pore size) before use. A guinea pig (300 g in body weight, male) was lightly anesthetized with diethyl ether and then the dye prepared was administered intravenously (65 mg per kilogram body weight), followed by the intradermal injection of 50 μl of vEP-45 (1 or 3 μg). After 10 min, the guinea pig was sacrificed and photographed to visualize the induced vascular permeability. For the quantification of the dye leakage, the skin around injection point (approximately one square centimeter) was cut out, soaked in 3 ml of formamide, and incubated for 48 h at 60 °C to allow the dye release. The amount of dye exclusion was determined by measuring the absorbance at 620 nm and expressed as a measure in μg of Evans blue dye efflux [6].

3. Results and discussion

3.1. vEP-45 can cleave and activate zymogens involved in contact system

The possible ability of vEP-45 to activate the zymogens involved in the intrinsic pathway of coagulation and the kallikrein/kinin system was first examined *in vitro* (Fig. 1). To see that polypeptide fragments capable of consisting active enzymes can be generated by vEP-45 cleavage, 10 μg each of zymogens (FXII, FXI, FX, and PPK) was digested with 0.2 μg of vEP-45 at 37 °C for 1 or 5 min and the resulting products were analyzed on 12% SDS–polyacrylamide gel (Fig. 1). As shown in Fig. 1A–D, polypeptide fragments comparable sizes to the chains comprising native enzymes seemed to be produced by vEP-45 cleavage from the corresponding zymogens, as indicated at the right sides of panels. These results suggest that vEP-45 may activate the zymogens through proteolysis as in the case of prothrombin activation [10]. Based on these results, the actual zymogen activation ability of vEP-45 was examined *in vitro* using chromogenic or fluorogenic peptide substrates specific for the activated enzymes (Fig. 1E–H). As shown in Fig. 1E, there was a clear increase in absorbance at 405 nm when 5 μg of FXII was digested with 0.2 μg of vEP-45 at 37 °C for 1 or 5 min and then 0.4 mM of S-2302 was added as a substrate, with no increase with zymogen only or vEP-45 alone. The relative FXIIa activity derived from vEP-45-cleaved FXII increased to an average of 9.07-folds, compared to that of non-cleaved zymogen (Fig. 1I). In this case, the adjusted unit of FXIIa activity was equivalent to approximately 0.96, where 1 unit was defined as an average absorbance value obtained with one μg of native enzyme. These results clearly show that vEP-45 can proteolytically activate FXII to active

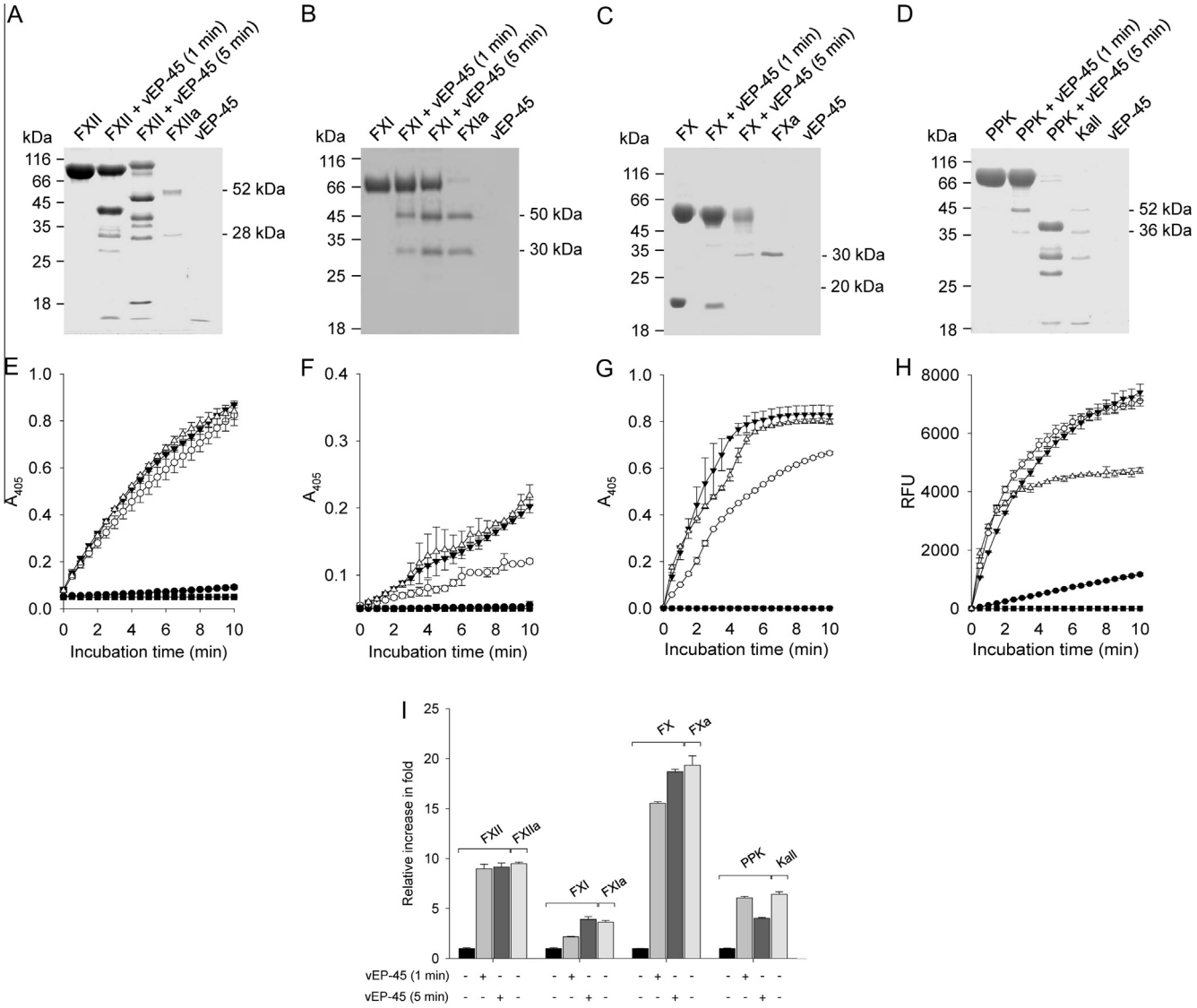

Fig. 1. Cleavage and activation of human zymogens involved in contact system by vEP-45. (A–D) The zymogens (each 10 μg), including FXII (A), FXI (B), FX (C), and PPK (D) were cleaved with vEP-45 (0.2 μg) at 37 °C for 1 min or 5 min and the resulting products were analyzed on 12% SDS–polyacrylamide gel, together with their active forms of zymogens (FXIIa, FXIa, FXa, and Kall; each 1 μg) and vEP-45 (0.2 μg). The sizes of polypeptides composed of the active enzymes are shown at the right sides of the panels. (E–H) The zymogens (each 5 μg), such as FXII (E), FXI (F), FX (G), and PPK (H) were cleaved with vEP-45 (0.2 μg) at 37 °C for 1 min or 5 min, and then 1,10-PT (1 mM) was added to inhibit the vEP-45 activity. Thereafter, the activities derived from vEP-45-cleaved zymogens were examined with 0.4 mM each of synthetic peptide substrates specific for the corresponding active enzymes as follows: S-2302 for FXIIa (E) and FXIa (F), S-2765 for FXa (G), and H-D-Val-Leu-Arg-AFC for Kall (H). As positive controls, the activities of the active enzymes (FXIIa, FXIa, FXa, and Kall; each 1 μg) were also analyzed with the same substrates. In the assays, the amidolytic activity was measured every 30 s at A_{405} for 10 min at 37 °C. The fluorescence was also monitored every 30 s at λ_{ex} = 400 nm and λ_{em} = 505 nm for 10 min at 37 °C, in which the activity was expressed as the relative fluorescence unit (RFU). Symbols: ■, vEP-45 only; ●, zymogen only (FXII, FXI, FX, or PPK); ○, corresponding zymogens plus vEP-45 (1 min); Δ, corresponding zymogens plus vEP-45 (5 min); ▼, corresponding active enzymes only (FXIIa, FXIa, FXa, or kallikrein). FXII, FXI, and FX mean the blood clotting factors XII, XI, and X, respectively. PPK and Kall indicate pre-kallikrein and kallikrein, respectively. (I) The enzyme activities derived from vEP-45-cleaved zymogens were calculated, for which the mean values ± S.D. of three independent experiments at the incubation periods of 10 min were expressed as relative increases in fold, compared to that of vEP-45-nontreated control.

FXII enzyme. As in FXII activation, the activation ability of vEP-45 could also be observed with other zymogens, including FXI, FX, and PPK, under the same experimental condition used for FXII (Fig. 1F–H). When S-2302 for FXIa (Fig. 1F), S-2765 for FXa (Fig. 1G), or H-D-Val-Leu-Arg-AFC for Kall (Fig. 1H) was used as a substrate for assaying vEP-45-induced activity, the relative fold increases were found to be 3.04 from FXI, 17.11 from FX, and 5.06 from PPK (Fig. 1I). In addition, the equivalent enzyme units of FXIa, FXa, and Kall were estimated to be 0.84, 0.88, and 0.79, respectively, where one unit was defined as an average absorbance value or a relative fluorescence unit (RFU) obtained with 1 μg of corresponding native enzyme. All these results indicate that vEP-45 exhibits a strong proteolytic activity to activate the zymogens involved in the intrinsic pathway of coagulation and the kallikrein/kinin system.

3.2. vEP-45 can activate the components of contact system in plasma milieu

The results obtained from *in vitro* experiments showed that vEP-45 could convert components of contact system to the active forms of enzymes (Fig. 1). Therefore, it was needed to confirm the vEP-45's ability capable of activating those zymogens present in blood plasma. To examine the ability, 10% of blood plasma was treated with 1 μg of vEP-45 for 1 or 5 min and then the induced activities of contact system components were observed

with 0.4 mM each of synthetic peptide substrates specific for the activated zymogens. When S-2302 was added to the vEP-45-treated plasma, the combined total activity of FXIIa and FXIa clearly increased to an average of 2.17-folds, compared to that of the substrate added only (Fig. 2A and E). However, a background level of increase could be observed with the same substrate when the enzyme was co-incubated with 1,10-PT, a potent inhibitor of vEP-45 [10,11] (Fig. 2A and E), suggesting that the increase of amidolytic activity is directly related to the vEP-45-cleaved activation of FXII and FXI zymogens. Likewise, vEP-45 also could activate other plasma zymogens, such as PPK (Fig. 2B), FX (Fig. 2C), and prothrombin (PT; Fig. 2D), in which the fold-increases of FXa, thrombin, and Kall activities were found to be 3.98, 8.95, and 6.19, respectively, compared to those of their corresponding substrates only added (Fig. 2E). However, the activations were leveled down, compared to those *in vitro* because the vEP-45 activity must have been inhibited by physiological inhibitors such as α_2-macroglobulin present in plasma [10]. All these results suggest that vEP-45 actually can convert inactive plasma FXII, FXI, FX, PT, and PPK zymogens to active FXIIa, FXIa, FXa, thrombin, and Kall, respectively, in plasma milieu.

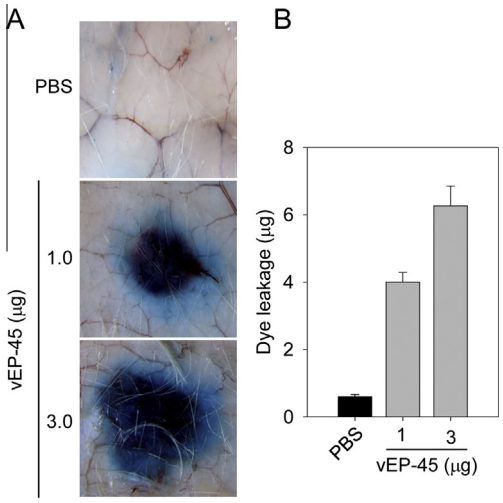

Fig. 3. The induction of vascular permeability by vEP-45. (A) PBS or vEP-45 (1 or 3 μg) was injected intradermally into a guinea pig that had been administered with Evans blue dye and then the vascular permeability was observed 10 min later. (B) The animal's back skins around the three injection sites were cut out, soaked in 100% formamide, and incubated for 48 h at 60 °C to allow the dye release. The amount of dye exclusion was determined by measuring the absorbance at 620 nm and expressed as a measure in μg of Evans blue dye efflux. HK, high-molecular weight kininogen; Kall, kallikrein.

3.3. vEP-45 may generate bradykinin by cleaving high-molecular weight kininogen and induces a vascular permeability in vivo

As described, the Kall activity could be observed from vEP-45-treated PPK and plasma (Figs. 1I and 2E). Accordingly, it was expected that the Kall enzyme generated by the protease could digest high-molecular weight kininogen (HK) to produce bradykinin (BK), which evokes the induction of a vascular permeability *in vivo*. In the cleavage assay, vEP-45 could cleave HK actively and produce various peptide fragments *in vitro* (data not

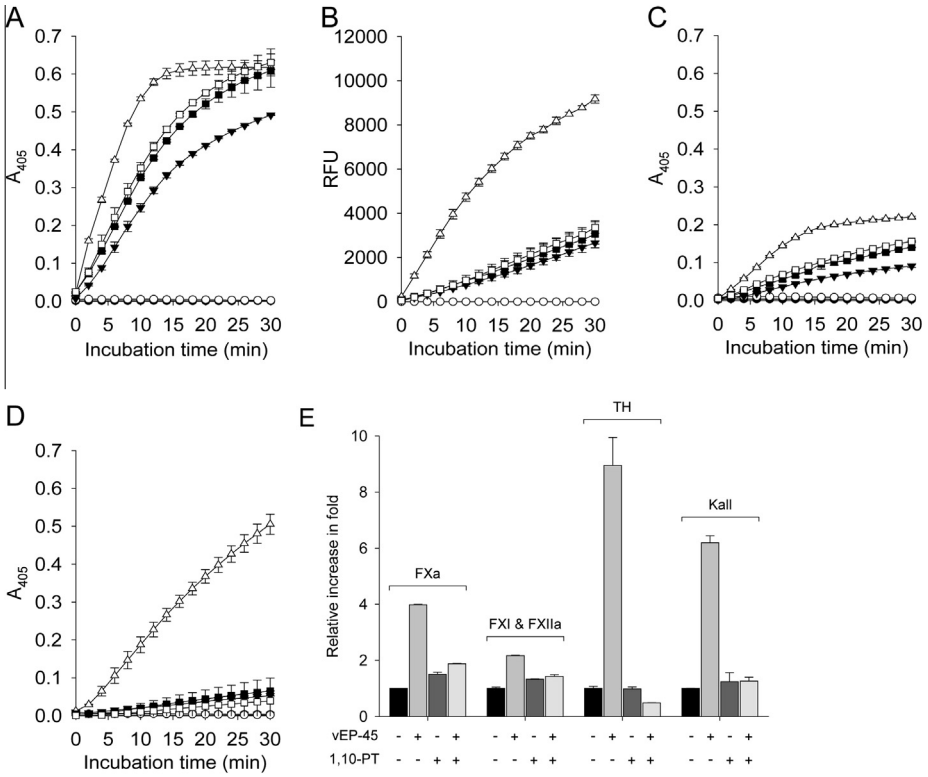

Fig. 2. vEP-45-induced activation of contact system components in plasma milieu. (A–D) Blood plasma (10%) was mixed with vEP-45 (1 μg) in the absence or presence of 1,10-PT (1 mM) and then various synthetic peptide substrates (each 0.4 mM), including S-2302 for assaying both FXIIa and FXIa activities (A), H-D-Val-Leu-Arg-AFC for Kall (B), S-2765 for FXa (C), and Val-Pro-Arg-*p*NA for thrombin (D) were added. The amidolytic activities and the fluorescence produced were then monitored every 2 min for 30 min at 37 °C as described already. Symbols: ●, plasma only; ○, vEP-45 only; ▼, corresponding peptide substrates added; Δ, corresponding peptide substrates plus vEP-45; ■, corresponding peptide substrates plus 1,10-PT; □, corresponding peptide substrates plus 1,10-PT plus vEP-45. (E) The enzyme activities derived from vEP-45-reacted plasma were calculated, for which the mean values ± S.D. of three independent experiments at the incubation periods of 10 min were expressed as relative increases in fold, compared to that of vEP-45-nontreated control.

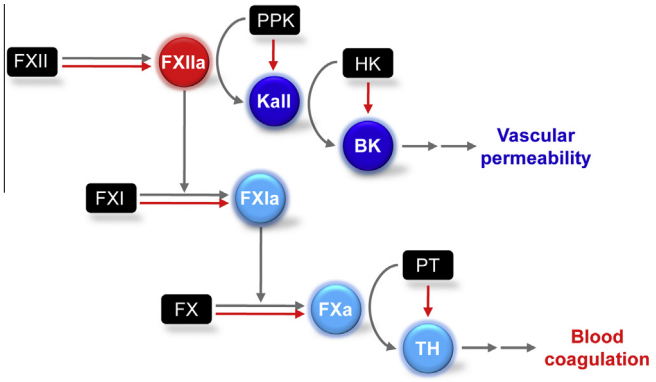

Fig. 4. Schematic representation of the contact system activation by vEP-45. The gray-colored arrows indicate the steps of endogenous activation pathway. The red-colored arrows represent the steps capable of being activated by vEP-45. The vascular permeability can be induced by vEP-45-activated and/or -generated enzymes and peptide(s) composed of kallikrein/kinin system, as shown by blue-colored circles. The intrinsic pathway of coagulation also can be triggered by vEP-45-produced FXIa, FXa, and TH, as shown by cyan-colored circles. The FXIIa controlling the cascade at the first step is shown with a red-colored circle. FXII, factor XII; FXI, factor XI; FX, factor X; PPK, plasma pre-kallikrein; Kall, kallikrein; HK, high-molecular weight kininogen; BK, bradykinin; PT, prothrombin; TH, thrombin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

shown), one of which might likely be BK though it could not see on the assay gel, due to its too small nonapeptide size to be observed [7].

These results suggest that the BK production can be occurred by vEP-45 through at least two ways: (i) the Kall enzyme generated by vEP-45-mediated PPK cleavage can make it by cleaving HK (Figs. 1B and 2B); (ii) vEP-45 may directly produce it by cleaving HK. In either event, the BK formed would act as a powerful mediator to induce a vascular permeability [2,7]. As shown in Fig. 3, the vEP-45 injection clearly resulted in the induction of vascular permeability, accompanied with the dye leakage in dose-dependent manner, as examined by Miles assay [14]. These results suggest that the vascular permeability and dye leakage can be induced and potentiated *in vivo* by the BKs generated by vEP-45-mediated cleavage of HK and/or -made Kall enzyme from PPK. However, it still cannot be ruled out a possibility that the vascular permeability can be also induced by mast cells activated by vEP-45 [15,16]. Therefore, this issue should be elucidated in further study.

3.4. Overall activation of contact system by vEP-45

It is been well known that Hageman factor (also known as FXII) acts as an initiator to trigger the intrinsic pathway of coagulation and the kallikrein/kinin system when it contacts to negatively charged surfaces or Poly P [1,4,16]. However, the results obtained show that the cascade of two systems can be initiated by FXIIa formed from vEP-45-cleaved and activated FXII. The vEP-45 protease can be more directly involved in the activation of the intrinsic pathway of coagulation through the production of FXIa, FXa, and thrombin (Figs. 1 and 2). In addition, vEP-45 can lead the activation of kallikrein/kinin system by cleaving PPK to make active Kall (Figs. 1H and 2B) as well as possibly by digesting HK to produce BK, which can induce a vascular permeability (Fig. 3). However, the generation of active FXIIa, FXIa, FXa, thrombin, and Kall by vEP-45 (Fig. 4) appeared to be transient, with further cleavage resulting in a loss of activity (data not shown), due to its broad-specificity on substrate proteins [10,11]. Taken together, the results obtained by the present study suggest that the progress of vibrio septicemia can be potentiated and accelerated by vEP-45 through its proteolytic activity capable of inducing blood coagulation and activating kallikrein/kinin system.

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