Induction of vascular leakage through release of bradykinin and a novel kinin by cysteine proteinases from *Staphylococcus aureus*

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*Staphylococcus aureus* is a major pathogen of gram-positive septic shock and frequently is associated with consumption of plasma kininogen. We examined the vascular leakage (VL) activity of two cysteine proteinases that are secreted by *S. aureus*. Proteolytically active staphopain A (ScpA) induced VL in a bradykinin (BK) B$_2$-receptor–dependent manner in guinea pig skin. This effect was augmented by staphopain B (SspB), which, by itself, had no VL activity. ScpA also produced VL activity from human plasma, apparently by acting directly on kininogens to release BK, which again was augmented significantly by SspB. Intravenous injection of ScpA into a guinea pig caused BK B$_2$-receptor–dependent hypotension. ScpA and SspB together induced the release of leucyl-methionyl-lysyl-BK, a novel kinin with VL and blood pressure–lowering activities that are equivalent to BK. Collectively, these data suggest that production of BK and leucyl-methionyl-lysyl-BK by staphopains is a new mechanism of *S. aureus* virulence and bacterial shock. Therefore, staphopain–specific inhibitors and kinin–receptor antagonists could be used to treat this disease.

Recent clinical studies revealed that gram-positive bacteria are as common as gram-negative bacteria in causing sepsis (1, 2) that often leads to septic shock, a condition with a high mortality, despite improved antibiotic therapy and intensive care. The pathogenesis of septic shock by gram-positive bacteria has not been elucidated fully. However, the plasma levels of the plasma kallikrein/kinin system components, factor XII, prekallikrein, and high molecular weight kininogen (HK), are low in patients who have sepsis (3–6); this indicates activation and subsequent consumption of these components. The activation of the plasma kallikrein/kinin system in an animal bacteremia model causes lethal hypotension (7, 8); hence, plasma kallikrein/kinin system activation seems to contribute to septic shock.

*Staphylococcus aureus* is the most frequently isolated pathogen in gram-positive sepsis (1, 9), which suggests that some factor from this bacterium is associated with septic shock induction. In human plasma, *S. aureus* induces the release of bradykinin (BK; reference 10), the final product of plasma kallikrein/kinin system activation which causes vascular leakage (VL; reference 11) and leads to hypotension. This bacterium has a high negative net surface charge because of the presence of cell wall teichoic acid and lipoteichoic acid (12), and can activate the plasma kallikrein/kinin system as efficiently as LPS and lipid A from gram-negative bacteria in vitro (13). Thus, these cell wall molecules also may activate the plasma kallikrein/kinin system in *S. aureus* bacteremia. However, heat-labile extracellular products of *S. aureus* are far more potent than the cell wall components as lethal factors in the mouse sepsis model (14).

In addition to enterotoxins and hemolysins, *S. aureus* secretes several extracellular proteinases (15) that may play a role in septic shock. The V8 proteinase can release kinin from HK. This activity is not abolished in the presence of serine proteinase inhibitors (16); this suggests that other than the V8 proteinase, contaminating proteolytic activity may have been responsible for kinin generation. Stapho-
pains A and B (ScpA and SspB) are *S. aureus*–derived cysteine proteinases. Their papainlike character has been investigated thoroughly with respect to molecular structure and post translational processing of proenzymes and led to the generation of 21-kD mature proteinases (17–20). Therefore, we purified these two cysteine proteinases from *S. aureus* culture medium and examined their VL and blood pressure (BP)–lowering activities. ScpA, especially in concert with SspB, possessed strong VL activity and lowered BP. The results indicate a new virulence mechanism in which staphopains liberate kinins, including a novel kinin that is released through alternative cleavage of kininogens. We believe that staphopains may be involved in septic shock that is caused by *S. aureus* infection.

**RESULTS**

**Induction of VL by ScpA**

ScpA induced VL in a dose–dependent manner starting at an enzyme concentration of 20 nM. In contrast to a linear increase of VL that is caused by exponentially increased doses of BK, the VL reaction that was triggered by ScpA injection increased steeply at higher enzyme concentrations (Figs. 1 and 2). Because ScpA showed no VL activity when inactivated by E-64, a cysteine proteinase inhibitor, the proteolytic activity of the enzyme is linked to production of VL activity (Figs. 1 and 2). Despite the lack of any significant VL activity, SspB increased ScpA–induced VL in a dose–dependent manner, whereas the proteinase exhibited no such effect on BK–induced VL (Fig. 2). HOE140, a BK B receptor antagonist, strongly inhibited VL that was induced by staphopains, BK, or histamine. Guinea pigs were injected with HOE140 (10 nmol/kg body weight) 30 min before intradermal injection of samples. (White bars) Without HOE140 treatment; (black bars) with HOE140 treatment. A, ScpA 300 nM; A+B, ScpA 60 nM + SspB 600 nM; BK, bradykinin 1 μM; HS, histamine 10 μM. Values are means ± SD (n = 3). *P < 0.01 for with versus without HOE140 treatment.

**Figure 2.** Leaked dye of each blue spot. (O), ScpA; (△), SspB; (●), BK; (▲), BK (100 nM) + SspB; (■), ScpA (60 nM) + SspB; (●), ScpA treated with E-64 (50 μM). *P < 0.01 for ScpA 200 nM versus 60 nM or 600 nM; for ScpA 60 nM + SspB 60 nM versus ScpA 60 nM + SspB 600 nM; and for BK 10 nM versus 100 nM. **P < 0.03 for BK 100 nM versus 1000 nM. Inset, the effect of HOE140 on VL activity that was induced by staphopains, BK, or histamine. Guinea pigs were injected with HOE140 (10 nmol/kg body weight) 30 min before intradermal injection of samples. (White bars) Without HOE140 treatment; (black bars) with HOE140 treatment. A, ScpA 300 nM; A+B, ScpA 60 nM + SspB 600 nM; BK, bradykinin 1 μM; HS, histamine 10 μM. Values are means ± SD (n = 3). *P < 0.01 for with versus without HOE140 treatment.

**Figure 3.** Leaked area vs. leaked dye amount. The spot area (mm²) was divided by the dye amount (μg) that was extracted from the skin (in μg). Although higher doses of BK and gingipain R, a bacterial proteinase that is known to elicit strong VL reaction (21), increased dye extravasation, the ratio of the blue
Production of VL activity from human plasma by ScpA

ScpA generated VL activity from normal human plasma in a dose- and activity-dependent manner when incubated for only 5 min (Fig. 4). SspB did not produce VL activity by itself; however, the enzyme significantly increased ScpA-induced VL activity production from human plasma (Fig. 4). The inhibitory effect of HOE140 on VL induction by direct ScpA injection into the guinea pig skin (Fig. 2, inset) and VL activity production from human plasma by ScpA, alone, or with both staphopains administered together (Fig. 4), indicated dependence of the VL reaction on the BK B₁ receptor. To identify the target protein of staphopains, we investigated the generation of VL activity by ScpA in plasmas that were deficient in factor XII, prekallikrein, or kininogens. ScpA produced VL activity from plasmas that were deficient in factor XII or prekallikrein, almost equivalent to the activity that was produced from normal plasma, but not from kininogen-deficient plasma (Fig. 4, inset). This suggested the direct action of ScpA on kininogen. To confirm this, we investigated the ScpA-dependent VL generation in kininogen-deficient plasma that was supplemented with physiologic concentrations of kininogens (22). ScpA induced VL activity from HK reconstituted kininogen-deficient plasma, which was increased further by the addition of low molecular weight kininogen (LK) to a level of the normal plasma concentration (Fig. 4, inset). Together, these data indicate that both kininogens are the major targets of staphopains, and activation of factor XII or prekallikrein contributes negligibly to the VL activity production.

Production of VL activity from human kininogens by staphopain A

To determine whether ScpA can produce VL activity from kininogens, we incubated purified human kininogens with the enzyme and measured VL activity. ScpA produced VL activity in a dose-dependent manner from both kininogens; it yielded more VL activity from LK than HK when used at their physiologic plasma concentrations (Fig. 5). SspB did not generate VL activity from HK, but augmented the ScpA-induced VL activity production in a dose-dependent manner (Fig. 5, inset). Consistent with previous results (Figs. 2, inset, and 4), HOE140 completely abolished VL activity that was generated from each kininogen by ScpA or by a combination of the two staphopains (Fig. 5). Preincubation of factor XII or prekallikrein with ScpA did not induce significant VL (Fig. 5) or hydrolysis of fluorogenic substrates.
that were specific for each activated form (not depicted); this indicated that ScpA does not activate these zymogens. These results indicate that staphopains cleave kininogens and release a peptide(s) that can induce VL through an interaction with the BK B₂-receptor. The ability of staphopains to cleave HK and LK was confirmed by SDS-PAGE analysis of the mixture of kininogens that were preincubated with staphopains at the weight ratio in the range from 50:1 to 5:1 (Fig. 6). LK was cleaved at the COOH terminus only by SspB, as recognized by a slight reduction of the molecular mass of the protein. Conversely, HK was degraded by both staphopains; SspB was more efficient than ScpA. In both cases, kininogen degradation occurred at molar excess of kininogens over the proteases in accordance with the lack of staphopain activity inhibition by kininogens (unpublished data). Collectively, the pattern and/or efficiency of the kininogen degradation corroborates well with VL activity generation by individual staphopains (Fig. 5), probably through kinin release.

Identification of VL activity released from HK by staphopains

To identify a VL factor(s) that was released from kininogens by ScpA, we separated peptides that were cleaved from HK by the proteinase and tested their VL activity. Only one peptide (VL factor-1) that eluted at the same retention time as BK had VL activity (Fig. 7). In addition, we found another VL peptide (VL factor-2), which eluted later than BK, in the HK sample that was preincubated with both staphopains; SspB was more efficient than ScpA. In both cases, kininogen degradation occurred at molar excess of kininogens over the proteases in accordance with the lack of staphopain activity inhibition by kininogens (unpublished data). Collectively, the pattern and/or efficiency of the kininogen degradation corroborates well with VL activity generation by individual staphopains (Fig. 5), probably through kinin release.

Blood pressure lowering by staphopains and kinins

To investigate further a link between staphopain-generated kinin release and septic shock, we compared the effect of staphopains and kinins on BP in guinea pigs. ScpA induced a β-branched amino acid at the P₂ position (unpublished data). Synthetic Leu–Met–Lys–BK exhibited almost equal VL activity as BK; the antagonist, HOE140, was able to inhibit its activity completely (Fig. 8). No VL was elicited by the scrambled peptide RGKRPLSFPFMP that has the same amino acid composition as Leu–Met–Lys–BK (Fig. 8).

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**Figure 6.** SDS-PAGE analysis of kininogen degradation by staphopains. 1 μg of LK (A and C) or HK (B and D) was incubated alone (lane 2), or with 0.02 μg (lane 3), 0.1 μg (lane 4), or 0.2 μg (lane 5) of ScpA (A and B) or SspB (C and D). After 4 h, reaction was stopped by addition of staphostatin (lane 6). (E) Purity of ScpA and SspB (lanes 7 and 8, respectively), loaded at 1 μg. Lane 1, molecular weight standards.

**Figure 7.** VL activity of HK fragments produced by staphopains. 180 μl of HK 280 μg/ml TBS was incubated with 20 μl of 1 μM ScpA (top), SspB (bottom), or both (middle) at 37°C for 10 min; fragments were separated with a C18 HPLC column. Each peak fraction was dried, dissolved in 100 μl of TBS, and examined for VL activity. Solid lines, dashed lines, and solid bars denote absorbance at 280 nm, acetonitrile gradient, and VL activity, respectively. Arrows indicate elution retention time of kallidin (KL) or bradykinin (BK), respectively. DL, dye leakage.
Figure 8. VL activity of LMK-BK and effect of HOE140 on the activity. (■, □), LMK-BK; (▲, △), BK; (●, ○), the scrambled peptide. Open and closed symbols denote VL activity in the absence or presence of HOE140 treatment. Values are means ± SD (n = 3). *P < 0.01 for BK 10 nM versus BK 1 or 100 nM and for LMK-BK 10 nM versus 100 nM. **P < 0.02 for LMK-BK 1 nM versus 10 nM.

Figure 9. Blood pressure lowering activity of ScpA (6 nmol/200 μl/kg body weight) and kinins (0.6 nmol/200 μl/kg body weight). (A) ScpA. (B) ScpA treated with E64 (50 μM). (C) ScpA in a guinea pig treated with HOE140 (10 nmol/kg body weight) 30 min before injection. (D) LMK-BK. (E) BK.

decrease of BP (by the mean BP 16.1 ± 5.3 mm Hg; n = 4) that peaked 15 s after injection and returned to the initial level 1 min later (Fig. 9 A). The effect was not induced by enzymatically inactive ScpA (Fig. 9 B), and was inhibited completely by HOE140 (Fig. 9 C). Synthetic Leu-Met-Lys-BK exhibited almost equivalent BP-lowering activity as BK (Fig. 8, D and E); they lowered the mean BP by 28.5 ± 2.4 mm Hg and 32.6 ± 2.9 mm Hg (n = 4), respectively. In contrast to the results of in vitro experiments, no significant augmentation of ScpA-induced BP-lowering effect was observed by simultaneous injection of SspB (unpublished data).

This most likely is due to immediate, more than 100-fold enzyme dilution in the bloodstream beyond the concentration that is needed for synergistic effect and/or SspB inhibition. Guinea pig plasma inhibitory activity for SspB is greater than for ScpA (unpublished data).

**DISCUSSION**

An important pathophysiologic mechanism of septic shock is hypovolemic hypotension that is caused by plasma leakage into the extravascular space. The fact that ScpA induced VL at a concentration as low as 20 nM within 5 min after injection into the guinea pig skin—with the reaction being augmented by coexisting SspB (Figs. 1 and 2)—indicates that VL induction by these proteinases can occur efficiently in vivo. The extensive spreading of plasma leakage that is caused by ScpA (Figs. 1 and 3) would facilitate further plasma loss into the extravascular space. Moreover, the fast generation of VL activity from human plasma by ScpA decreases the chance of enzyme clearance from the circulation and suggests that these proteinases may cause septic shock in cases of severe human *S. aureus* infection. The dependency of staphopain VL activity on the BK B2-receptor (Figs. 2, 4, and 5), or the presence of kininogen (Fig. 4), clearly showed that this pathogenic activity is exerted by kinin production, which is one of the prominent features of septic shock (3–8).

Furthermore, because staphopains also can act on LK—whose plasma molar concentration is threefold greater than HK (Fig. 5; reference 22)—they also have more opportunity to interact with substrate than proteinases that generate BK only from HK. Taken together, these results indicate that VL induction by staphopains could be a new mechanism of septic shock induction in severe *S. aureus* infection. This contention is supported by the BP-lowering effect of ScpA, which is dependent on the proteolytic activity of the enzyme and the BK B2-receptor (Fig. 9).

The premise gains further credence in light of a study that showed that heat-labile extracellular products of *S. aureus*, but not heat-treated bacterium, exerted lethal activity in a mouse sepsis model (14). This strongly argues against the importance of heat-stable components of the *S. aureus* cell wall, including teichoic acid and lipoteichoic acid as causative molecules of septic shock, despite the fact that they can activate the plasma kallikrein/kinin system (10). Moreover, in the mouse model, the lethal event was not dependent on toxins, such as toxic shock syndrome toxin 1, enterotoxins (A, B, and D), or hemolysins (α, β, and γ; reference 14). This implicates other proteins that are secreted by *S. aureus*—especially proteolytic enzymes—including epidermolytic toxins; the metalloproteinase, aureolysin; the V8 protease; and the two staphopains. The epidermolytic toxins, which are serine proteinases and prefer glutamic acid at the P1 site, cause epidermal dissociation, the pathologic hallmark of bullous impetigo and staphylococcal scalded-skin syndrome; however, they do not trigger edema when injected into newborn mouse skin (23) or guinea pig skin (unpublished data), so they are unlikely to cause lethal sepsis. Conversely, hand-purified aureolysin and the V8 protease did not increase vascular permeability, even at 3 μM (unpublished data). This leaves staphopains as the only major VL factors of *S. aureus* that are responsible for septic shock induction.

Kinin-releasing cysteine proteinases have been reported from various sources, including cruzipain from *Trypanosoma*...
The promotion of leaked plasma that is spread by ScpA (Figs. 1 and 3) may be due to connective tissue damage through degradation of elastin (31), and possibly other extracellular matrix proteins by ScpA. Another bacterial protease, gingipain R, does not have any elastinolytic activity (not depicted) and the dye spread that accompanied the VL reaction did not occur (Fig. 3). Alternatively, the effect can be exerted by loosening a tight contact between epithelial cells. Exposure of ScpA to a human airway epithelial cell line resulted in a dramatic loss of intercellular adhesion of the monolayer in an enzymatic activity-dependent manner (unpublished data). It is likely that ScpA causes destruction of the interstitial tissue, which is filled with cells and substances including adhesion proteins, bringing storage space of leaked plasma. This may explain, in part, the steep dose-dependent increase of ScpA VL activity (Fig. 2), whereas the VL is mediated by B2-receptor as is the VL by BK, whose VL activity increases linearly (Fig. 2).

Conclusively, staphopains that are secreted from S. aureus in the infected sites or in the circulation, are likely to produce BK and Leu-Met-Lys-BK, and lead to septic shock through their VL activity. This may be a new virulence mechanism for this bacterium. The fact that the BP-lowering activity of ScpA was not augmented by SspB in guinea pig does not exclude the possibility that Leu-Met-Lys-BK can be formed in human plasma devoid of SspB inhibitory activity. Because Leu-Met-Lys-BK exerted comparative BP-lowering activity with BK (Fig. 8), we suggest that the release of this new kinin is a key event in the development of septic shock by S. aureus. Therefore, staphopains may constitute important therapeutic targets for S. aureus septic shock and inhibitors of these enzymes—together with BK B2-receptor antagonists—could be developed as drugs, particularly against antibiotic-resistant strains (e.g., methicillin-resistant S. aureus).

**MATERIALS AND METHODS**

**Materials.** Human HK, factor XII, and prekallikrein were purchased from Enzyme Research Laboratories. LK was purchased from Athens Research Technology. Evans blue was obtained from Merck. Soybean trypsin inhibitor (SBTI) was purchased from Sigma-Aldrich. BK, kallidin, and E-64, a cysteine proteinase inhibitor, were obtained from the Peptide Institute. BK B2-receptor antagonist, HOE140, was obtained from Hoechst AG. Other chemicals were purchased from Wako Pure Chemicals. Plasmas that were deficient in factor XII, prekallikrein, or kininogens were purchased from George King Bio-Medical, Inc. Normal human plasma was prepared by centrifugation of a mixture of nine volumes of freshly drawn blood from healthy volunteers and one volume of 3.8% (wt/vol) sodium citrate.

Purification of staphopains and titration of their enzymatic activity. Staphopains were purified from the culture media supernatants of S. aureus strain V8-BC10 or 8325-4 as described previously (31). Purity of staphopains was assessed by SDS-PAGE, mass spectroscopy, and NH2-terminal amino acid sequence analysis. The enzymes were shown to be homogenous with a molecular mass of 21 kDa (Fig. 6, panel E); this matched the mass that was calculated from amino acid composition inferred from the gene structure and known processing site of proenzymes (32). The active site concentration of ScpA and SspB was determined by enzyme titration with E-64 and staphostatins, respectively (31, 33).

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Treatment of plasmas and kinogens with staphopains. 45 μl of normal human plasma or plasma that was deficient in factor XII, prekallikrein, or kinogens and supplemented with 1 mM 1,10-phenanthroline to inhibit kininases was incubated with 5 μl of various concentrations of a staphopain at 37°C for 5 min, followed by the addition of 50 μl of 10 mM Tris-HCl, pH 7.3, containing 150 mM NaCl (Tris-buffered saline [TBS]) supplemented with 0.1 mM E-64, 1 mM 1,10-phenanthroline, and 20 μg SBTI. 10 μl of staphopain and 90 μl of TBS which contained HK (80 μg/μl), LK (130 μg/μl), or both was incubated at 37°C for 10 min; 5 μl of 1 mM E-64 was added to stop the reaction.

Measurement of staphopain activity inhibition by kinogens. Staphopains at 10 nM final active enzyme concentration, as determined by active site titration, were preincubated with 10-fold molar excess of LK or HK for 15, 60, and 120 min at 37°C. The residual enzyme activity was assayed with azocoll or synthetic fluorogenic substrates as described previously (18, 20, 33).

SDS-PAGE analysis of kinogen degradation by staphopains. 1 μg of human LK or HK was incubated alone (controls) or with different amounts (0.02–0.2 μg) of purified staphopains in a total volume of 20 μl of TBS for 4 h at 37°C. The reaction was stopped by the addition of 2 μg of staphokinase, which are specific and very effective inhibitors of staphopains (33). Samples were boiled under reducing conditions and analyzed using SDS-PAGE (12% polyacrylamide gels; reference 34). Gels were silver stained.

VL assay. This experiment was performed according to the criteria of animal experiments of Kumamoto University Animal Experiment Committee and was permitted by the Committee. Guinea pigs (~350–450 g body weight, both sexes) were anesthetized with an intramuscular injection of ketamine (80 mg/kg body weight). 30 mg/kg body weight of Evans blue (2.5% solution in 0.6% saline) was administered intravenously, followed by an intradermal injection of 0.1 ml of test sample (dissolved in 10 mM PBS) into the clipped flank of the guinea pig. After 10 min the guinea pig was killed by bleeding; bluing tissues were cut out and incubated in 3 ml of formamide at 60°C for 48 h. VL activity was determined by quantitatively measuring the extracted Evans blue by absorbance at 620 nm as described previously (21). Activity was expressed in terms of μg of dye extracted. The activity of the buffer was subtracted from the activity of each sample. HOE140 was injected subcutaneously to a guinea pig (10 nmol/kg body weight) 30 min before intradermal injection of samples.

Measurement of dye leaked area. The length (A) and width (B) of a blue spot were measured. The area was calculated by A/2 × B/2 × 3.14.

Measurement of blood pressure. Guinea pigs (~350–400 g body weight, both sexes) were anesthetized with an intramuscular injection of ketamine (100 mg/kg body weight) and ether inhalation. A blood pressure transducer (Mikro-Tip catheter transducer model SPR-671), connected to a transducer amplifier (transducer control unit model TCB-500, Millar Instruments) with a recorder (miniwriter WR7200, GRAPHTEC), was inserted into the left common carotid artery. Samples diluted with PBS were administered in a single bolus injection into the left femoral vein.

Peptide separation. 180 μl of HK (280 μg/ml TBS) was incubated with 20 μl of 1 μM staphopain A at 37°C for 10 min, followed by the addition of 10 μl of 1 mM E-64. An aliquot of the reaction mixture was applied on a C18 HPLC column (4.6 x 150 mm; Yamaanura Chemical Laboratories Co. Ltd.) equilibrated with 0.1% TFA containing 16% acetonitrile, and eluted by a linear gradient of acetonitrile to 24% for 30 min at a flow rate of 0.5 ml/min. The HPLC was performed with a Hitachi model 655A; effluents were monitored at 210 nm with a Hitachi model 655A-21 UV monitor. Each peak fraction was dried, dissolved in 100 μl of TBS, and examined for VL activity.

Amino acid sequence determination. Separated peptide peaks with VL activity were subjected to an automated peptide sequencer (model 477A; Perkin-Elmer/Applied Biosystems) equipped with an online phenylthiohydantoin analyzer (model 20A; Perkin-Elmer/Applied Biosystems).

Peptide synthesis. LMKRPGFSFPR (Leu-Met-Lys-BK) and its scrambled peptide, RGRKLPSFPFEP, were synthesized by the standard t-Boc method using a solid-phase peptide synthesizer (model 430A; Perkin-Elmer/Applied Biosystems) and purified by HPLC with a C8 column after cleavage from the solid support and deprotection. Purity of the peptide (>98%) was determined by HPLC and amino acid sequence analysis.

Statistics. Statistical analysis was performed using unpaired Student’s t test. Values were expressed as means ± SD in triplicate assay.

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