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Induction of Vascular Leakage and Blood Pressure Lowering through Kinin Release by a Serine Proteinase from *Aeromonas sobria*

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*Aeromonas sobria* causes septic shock, a condition associated with high mortality. To study the mechanism of septic shock by *A. sobria* infection, we examined the vascular leakage (VL) activity of *A. sobria* serine proteinase (ASP), a serine proteinase secreted by this pathogen. Proteolytically active ASP induced VL mainly in a bradykinin (BK) B$_2$ receptor-, and partially in a histamine-H$_1$ receptor-dependent manner in guinea pig skin. The ASP VL activity peaked at 10 min to 1.8-fold of the initial activity with an increased BK B$_2$ receptor dependency, and attenuated almost completely within 30 min. ASP produced VL activity from human plasma apparently through kallikrein/kinin system activation, suggesting that ASP can generate kinin in humans. Consistent with the finding that a major part of the ASP-induced VL was reduced by a potent kallikrein inhibitor, soybean trypsin inhibitor that does not affect ASP enzymatic activity, ASP activated prekallikrein but not factor XII to generate kallikrein in a dose- and incubation time-dependent manner. ASP produced more VL activity directly from human low m.w. kininogen than high m.w. kininogen when both were used at their normal plasma concentrations. Intraarterial injection of ASP into guinea pigs lowered blood pressure specifically via the BK B$_2$ receptor. These data suggest that ASP induces VL through prekallikrein activation and direct kinin release from kininogens, which is a previously undescribed mechanism of *A. sobria* virulence and could be associated with the induction of septic shock by infection with this bacterium. ASP-specific inhibitors, and kinin receptor antagonists, might prove useful for the treatment or prevention of this fatal disease.


*Aeromonas* species are facultative anaerobic Gram-negative rods that are ubiquitous, waterborne bacilli (1), most commonly implicated as causative agents of gastroenteritis (2–5). *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas sobria* recovered predominantly from clinical samples have been reported to be involved in a wide range of extraintestinal and systemic infections, including septicemia, wound infections, meningitis, peritonitis, and hepatobiliary disease (6). The majority of patients infected with *Aeromonas* septicemia are infants under 2 years of age and immunocompromised adults with multiple underlying medical complications, mostly malignancy, hepatobiliary disease, or diabetes. Mortality rates for these patients generally range from 25 to 50% (7–9). Patients with severe wound infection (myonecrosis) caused by this microorganism also develop sepsis, and >90% of the patients succumb to their infections (7, 10). Septic shock is a major cause of death of these patients, but no mechanism leading to this fatal complication has been shown in *Aeromonas* infections. Understanding the mechanism of *Aeromonas* septic shock is crucial in the development of therapeutic treatments. Recently, overactivation of the immune system by the excessive release of proinflammatory cytokines, e.g., TNF-α, mainly from monocytes/macrophages stimulated by bacterial lipopolysaccharides through Toll-like receptors, a class of pattern recognition molecules, have been suggested to lead to septic shock in Gram-negative bacteria infections (11). However, the fact that impairment of the host immune system increases the incidence of *Aeromonas* septic shock (6) may indicate involvement of a mechanism(s) other than overactivation of the immune system in shock induction.

The plasma kallikrein/kinin system is initiated by activation of coagulation factor XII on a negatively charged surface, with activated factor XII converting plasma prekallikrein to kallikrein, releasing bradykinin (BK)$^\dagger$ from high m.w. kininogen (HK) (12). The plasma levels of the kallikrein/kinin system components are low in sepsis patients (13–16), indicating the activation and subsequent consumption of these components. BK, the final product, binds to BK-B$_2$ receptor on vascular endothelial cells (17) and induces vascular leakage (VL), leading to hypotension due to a decrease in circulating blood volume. Indeed, the activation of the plasma kallikrein/kinin system in an animal model causes lethal hypotension (18, 19). Hence, activation of the kallikrein/kinin system, and/or direct kinin release from kininogens, seems to contribute to septic shock.

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$^\dagger$Abbreviations used in this paper: BK, bradykinin; HK, high m.w. kininogen; VL, vascular leakage; LK, low m.w. kininogen; ASP, *Aeromonas sobria* serine proteinase; BP, blood pressure; SBTI, soybean trypsin inhibitor; DFP, diisopropyl fluorophosphate; ScpA, staphopain A.
Aeromonas species release a number of putative virulence factors including hemolysins, enterotoxins, and proteinases (20). We have shown that bacterial proteinases induce VL through activation of the plasma kallikrein/kinin system, and/or direct kinin release from HK and low m.w. kininogen (LK), which represents another kinin source in plasma (21–23).

A. sobria is predominantly isolated in patient’s blood (24), and is more pathogenic than another kinin source in plasma (21–23).

Materials and Methods

Human HK, factor XII, and prekallikrein were purchased from Enzyme Research Laboratories, and human LK was obtained from Athens Research Technology. Evans blue was obtained from Merck. Soybean trypsin inhibitor (SBTI) was purchased from Sigma-Aldrich. BK, benzyloxycarbonyl-L-phenylalanly-l-arginine-4-methylcoumaryl-7-amide (Z-Phe-Arg-MCA), and t-butylxocarbonyl-L-glutaminylglycyl-l-arginine-4-methylcoumaryl-7-amide (Boc-Gln-Gly-Arg-MCA) were obtained from the Peptide Institute, BK B2 receptor antagonist, HOE140 was obtained from Hoechst. 7-amide (Boc-Gln-Gly-Arg-MCA) were obtained from the Peptide Institute. 37°C with shaking for 20 h. ASP secreted into the culture supernatant was purified by successive column chromatography (27). The ASP sample was analyzed by SDS-PAGE under reducing conditions using an 8% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. Kininogens were blotted with anti-human kallikrein IgG rabbit IgG, which interfere with kininogen immunoblotting. Albumin binds non-specifically measuring the extracted Evans blue by absorbance at 620 nm as described previously (30). Activity was expressed in terms of micrograms of dye extracted. The activity of the buffer was subtracted from the activity of each sample. In the case of VL activity production from human plasma, the activity of plasma incubated with the buffer, followed by DFP addition, was subtracted from the activity of each sample. HOE140 (10 nm/kg body weight) or diphenhydramine (30 mg/kg body weight) dissolved in 200 µl of TBS was injected s.c. in the thigh at 30 min, or i.p. in the lower abdomen at 1 h, respectively, before intradermal injection of samples into guinea pigs.

Measurement of activation of prekallikrein or factor XII by ASP

Several million of prekallikrein (1 µM) or factor XII (1 µM) in 0.1 M Tris-HCl buffer (pH 7.6) containing 150 mM NaCl was incubated with 10 µl of ASP at 37°C for a range of different time periods, followed by an additional 500 µl of the buffer preincubated at 37°C. Then, after the addition of 10 µl of 10 mM Z-Phe-Arg-MCA (plasma kallikrein-specific substrate) (28) or Boc-Gln-Gly-Arg-MCA (activated factor XII-specific substrate) (29), the 7-amino-4-methyl coumarin released in the solution was measured (fluorescence at 440 nm with excitation at 380 nm) using a fluorescence spectrophotometer (model no. 650-40; Hitachi).

Treatment of plasmas and kininogens with ASP

Forty-five microliters of human plasma supplemented with 1 mM 1,10-phenanthroline for kininase inhibition was incubated with 5 µl of ASP at various concentrations and 37°C for 5 min, followed by the addition of 50 µl of 10 mM Tris-HCl (pH 7.3) containing 150 mM NaCl (TBS) supplemented with 1 mM 1,10-phenanthroline. Ten microliters of ASP and 90 µl of TBS containing HK (80 µg/ml) or LK (130 µg/ml) were incubated at 37°C for 5 min. One µl of 100 mM diisopropyl fluorophosphate (DFP) was added to plasma or kininogen samples after incubating with ASP to completely inhibit ASP (27). DFP specifically phosphorylates the active-site serine residue of serine proteinases, inactivating its enzymatic activity.

VL assay

This experiment was performed and approved according to the criteria of animal experiments of the Kumamoto University Animal Experiment Committee. Guinea pigs (350–450 g body weight, both sexes) were anesthetized with an i.m. injection of ketamine (80 mg/kg body weight). Thirty mg/kg body weight of Evans blue (2.5% solution in 0.6% saline) was then administered i.v., followed by an intradermal injection of 0.1 ml of test samples (dissolved in TBS) into the clipped flank of the guinea pig. Bluing of injection sites starts within a few minutes and terminates by 10 min. After 10 min, the guinea pig was euthanized by bleeding under ether anesthesia and blue dye-leaked skin tissues were excised, and incubated in 3 ml of formalin 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 (30).

Immunoblot analysis of ASP-treated human plasma

Ten microliters of citrated human plasma, supplemented with SBTI (0.1 mM) to inhibit kininogen cleavage by kallikrein, was incubated with 1 µl of ASP (1 µM) at 37°C for 30 min, followed by the addition of 0.1 µl of DFP (0.1 M) to inactivate ASP. Plasma was then diluted with 60 µl of 25 mM Tris-HCl buffer (pH 7.5) containing 25 mM NaCl and mixed with 170 µl of immobilized Cibacron blue/protein A gel (Pierce) to remove albumin and IgG, which interfere with kininogen immunoblotting. Albumin binds specifically to Cibacron blue and IgG to protein A. A control, plasma was processed as described above except ASP treatment was used. As further controls, HK (80 µg/ml) and LK (130 µg/ml) were incubated with 30 nM ASP or TBS at 37°C for 10 min, followed by the addition of 1 mM DFP. Kininogen samples were then analyzed by SDS-PAGE under reducing conditions using an 8% polyacrylamide gel. Each Gel was silver stained using the Silver Stain II kit (Wako Pure Chemical Industries).

Measurement of BP

Guinea pigs (350–400 g body weight, both sexes) were anesthetized by an i.m. injection of ketamine (100 mg/kg body weight) and ether inhalation.

FIGURE 1. SDS-PAGE of ASP. ASP (0.6 µg) was analyzed using a SDS-polyacrylamide gel in the presence (lane 3) or absence of 2-ME (lanes 1 and 2), and the gel was resolved by silver-staining. Lane 1, Molecular size markers; lanes 2 and 3, ASP.
A BP transducer (MIKRO-TIP catheter transducer model SPR-671) connected to a transducer amplifier (transducer control unit model TCB-500; Millar Instruments) with a recorder (minirewriter WR7200; Graphitec) was inserted into the right carotid artery. A hundred microliters of ASP diluted with TBS was administered in a single bolus injection into the left ventricle. HOE140 (10 nm/kg body weight) dissolved in 200 μl of TBS was injected s.c. at 30 min before sample injections into guinea pigs. Representative results were shown.

Statistics

Statistical analysis was performed using an unpaired Student’s t test. Values were expressed with means ± SD (n = 3 or 4).

Results

Induction of VL by ASP

ASP used in this study was proved to be homogenous, showing a single band under both conditions on a SDS-PAGE gel (Fig. 1). First, VL activity of ASP was studied in guinea pigs. ASP induced VL in a dose-dependent manner starting at an enzyme concentration of 30 nM (Fig. 2A). In contrast to a linear increase of VL caused by exponentially increasing doses of BK, the VL reaction triggered by ASP injection increased steeply at higher enzyme concentrations (Fig. 2, A and B). Inactivation of ASP by the serine protease inhibitor DFP resulted in the loss of VL activity (Fig. 2, A and B), suggesting that the proteolytic activity of the enzyme is linked to the induction of VL. Interestingly, SBTI reduced >60% of the ASP-induced VL (Fig. 2B), while not affecting ASP enzymatic activity (27). The ASP-induced VL was mostly inhibited by HOE140, a BK B2 receptor-specific antagonist (31, 32), and partially by diphenhydramine, a histamine H1 receptor antagonist (Fig. 2B, inset). The sensitivity to SBTI, a potent inhibitor for plasma kallikrein (33, 34), and the BK B2 receptor dependency of ASP VL activity together suggests an involvement of kinin release through activation of the plasma kallikrein/kinin system in ASP-induced VL. The ASP VL activity increased to 1.8-fold 10 min after intradermal ASP injection in comparison with the initial VL activity and reduced to the base level almost completely at 30 min (Fig. 3). Through the time course HOE140 strongly inhibited ASP-induced VL, and the BK B2 receptor antagonist abolished ~90% of the maximal VL at 10 min (Fig. 3).

Production of VL activity from human plasma by ASP

To study the possibility that ASP can produce VL activity in humans, ASP was incubated with human plasma, treated with DFP, and the VL activity of the plasma was measured. ASP produced VL activity from human plasma in a dose- and enzymatic activity-dependent manner, with ~40% of the ASP VL activity production being inhibited by SBTI (Fig. 4). HOE140 and diphenhydramine...
abolished the VL activity by ~60 and 40%, respectively (Fig. 4). These results suggest that ASP can induce VL in humans mainly through the BK B2 receptor, and partially via the histamine H1 receptor.

**Activation of human prekallikrein by ASP**

The observation that SBTI inhibited mostly the BK B2 receptor-dependent VL activity of ASP-treated human plasma (Fig. 4), suggests a mediation of activation of human factor XII and/or prekallikrein by ASP. To investigate the involvement of this pathway, purified human prekallikrein or factor XII was incubated with ASP, and the enzymatic activities were measured with synthetic substrates specific to each activated form. ASP generated kallikrein activity from human prekallikrein in a dose- and incubation time-dependent manner, but no enzymatic activity was detected from human factor XII incubated with ASP (Fig. 5, A and B). No kallikrein activity was generated when ASP was inactivated with DFP (Fig. 5, A and B), and ASP hydrolyzed neither of the synthetic substrates. These results indicate a mechanism of VL induction by ASP, because ASP activates only prekallikrein, and the product, kallikrein, releases BK from HK.

**Production of VL activity from human kininogens by ASP**

The observation that SBTI did not inhibit BK B2 receptor-dependent VL activity of ASP-treated human plasma (Fig. 4) prompted us to investigate direct VL activity production from kininogens by ASP. ASP was incubated with either of the purified human kininogens, and VL activity was measured upon the inactivation of ASP with DFP. ASP produced VL activity from both kininogens; it yielded 2- to 3-fold more VL activity from LK than HK when these kininogens were used at their physiological plasma concentrations (35) (Fig. 6). Because the kininogen-derived VL activity was completely inhibited by HOE140 (Fig. 6), the activity is mediated by kinin generation. The VL activity production from kininogens by ASP, with a preference for LK rather than HK under physiological conditions indicates another mechanism of ASP VL induction. To investigate kininogen cleavage by ASP in plasma, human plasma was incubated with ASP and analyzed for kininogen fragments by immunoblotting. HK (110 kDa) and LK (64 kDa) were present in the initial plasma samples (Fig. 7A, lane 1), with the two kininogens converted into three fragments in ASP-treated plasma (Fig. 7A, lane 2). From the mobility of the fragments from ASP-treated kininogens (Fig. 7B, lanes 2 and 4), fragments of 47 and 58 kDa appear to be derived from both kininogens and 32-kDa fragment from HK (Fig. 7). These kininogen fragment bands detailed in the immunoblotting correspond well to those observed under SDS-PAGE analysis (Fig. 7B). The finding that ASP can cleave kininogens in plasma supports the VL activity production ability of ASP in humans, which might contribute to the septic shock genesis through kinin release.

**BP lowering by ASP**

The kinin generation activity of ASP suggests that this proteinase can result in the hypotension seen in septic shock patients. To study a link between ASP and septic shock further, we investigated the effect of ASP on BP in guinea pigs. ASP induced a drop of BP (by the mean BP 20.3 ± 2.7 mmHg; n = 4), which peaked 15 s after injection, and returned to initial levels 90 s later (Fig. 8A). The effect was not induced by enzymatically inactive ASP (by the mean BP 3.0 ± 3.3 mmHg; n = 4; p < 0.0002) (Fig. 8B). Notably, the ASP BP-lowering activity was

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**FIGURE 5.** Activation of prekallikrein by ASP. One micromolar prekallikrein (○) or factor XII (△) was incubated with ASP at 37°C for various periods, and the amidolytic activity for a fluorogenic substrate specific for kallikrein or activated factor XII, respectively, was measured. A, Incubated with various concentrations of ASP for 5 min. B, Incubated with 10 nM ASP for various periods. ●, Prekallikrein incubated with DFP-treated ASP.

**FIGURE 6.** Production of VL activity from kininogens by ASP. HK (80 μg/ml) or LK (130 μg/ml) was incubated with various concentrations of ASP at 37°C for 5 min, followed by 1 mM DFP addition. VL activity was then measured in guinea pigs treated with or without HOE140. (○), HK; (△), LK. Closed symbols indicate the activity in HOE140-treated animals. Values are means ± SD (n = 3). *, p < 0.01 for HK + ASP 3 nM vs + ASP 30 nM; and for HK vs LK. **, p < 0.02 for LK + ASP 3 nM vs + 30 nM.

**FIGURE 7.** Kininogen cleavage in plasma by ASP. Human plasma was incubated with ASP and after treating with immobilized Cibacron blue/protein A gel, the plasma was applied to a SDS-polyacrylamide gel under reducing conditions. Kininogen fragments were then analyzed by immunoblotting (A, lanes 1 and 2). The result of immunoblotting (A, lanes 3–6) of ASP-treated kininogens was shown together with the result of SDS-PAGE analysis (B, lanes 1–4). A, Immunoblotting: lane 1, plasma; lane 2, ASP-treated plasma; lane 3, HK (0.1 μg); lane 4, ASP-treated HK (0.1 μg); lane 5, LK (0.1 μg); lane 6, ASP-treated LK (0.1 μg). B, SDS-PAGE: lane 1, HK (1 μg); lane 2, ASP-treated HK (1 μg); lane 3, LK (1 μg); lane 4, ASP-treated LK (1 μg).
inhibited almost completely by HOE140 (by the mean BP 4.1 ± 2.5 mmHg; n = 4; p < 0.0002) (Fig. 8C). The result suggests that ASP in the bloodstream lowers BP in an enzymatic activity-dependent manner, and almost all of the activity is mediated by kinin production.

Discussion

An important pathophysiological mechanism of septic shock is hypovolemic hypotension caused by plasma leakage into the extravascular space. The fact that ASP induced VL at a concentration as low as 30 nM, 5 min postinjection into guinea pig extravascular space. The fact that ASP induced VL at a concentration as low as 30 nM, 5 min postinjection into guinea pig skin (Fig. 2, A and B) indicates that VL induction by this enzyme can occur efficiently in vivo. Moreover, the rapid generation of VL activity from human plasma by ASP (Fig. 4) decreases the likelihood of enzyme clearance from the circulation, and suggests that this proteinase may cause septic shock in human cases of severe A. sobria infection. The dependency of ASP VL activity on the BK B2 receptor (Figs. 2B, inset, 3, and 4) clearly show that this pathogenic activity is exerted by kinase production, which is one of the prominent features of septic shock (13–16). Because ASP activates prekallikrein (Fig. 5), an upstream component of the proteolytic cascade reaction of the plasma kallikrein/kinin system (12), this facilitates efficient kinin production by plasma kallikrein, the biological BK liberator from HK. Furthermore, because ASP can also act on LK (Figs. 6 and 7), whose plasma molar concentration is 3-fold higher than that of HK (35), this proteinase also has an opportunity to interact with more substrates than proteinases that generate BK only from HK. Taken together, these results indicate that VL induction by ASP could be an underlying mechanism of septic shock induction in severe A. sobria infection. This contention is supported by the BP-lowering effect of ASP, which is dependent on both the proteolytic activity of the enzyme and the BK B2 receptor (Fig. 8).

The dominant inhibitory effect of HOE140 on ASP VL (Figs. 2B, inset, 3, and 4) indicates that kinase release contributes mainly to the VL. VL by ASP and by ASP-treated human plasma was inhibited by SBTI more effectively than by diphenhydramine, and the VL inhibition ratios of SBTI vs those of HOE140 were >60% (Figs. 2B, inset, and 4). It is likely that most of the ASP B2 receptor-dependent VL is caused by BK liberated by kallikrein converted from plasma prekallikrein (Fig. 5) with the rest mediated by direct kinin release from kininogens (Fig. 6). The substrate specificity of ASP, which demonstrates a strong preference for basic amino acid at the P1 position (36), is consistent with the cleavage sites requisite for prekallikrein conversion to kallikrein (-Arg-→Ile-) (37) and for kinin release from kininogens (-Arg-→Ser-) (38), supporting these ASP abilities. Human prekallikrein activation has been shown for cysteine proteinases (gingipains-R) produced by Porphyromonas gingivalis (21, 39), the major pathogen of adult periodontitis, and a metalloprotease from Vibrio vulnificus (40).

In accordance with the case of ASP (Fig. 2B), SBTI also inhibited VL by these proteinases without affecting their enzymatic activities (21, 41). No serine protease of bacteria origin has been reported as a prekallikrein activator; hence, ASP is thus far the only serine proteinase shown to possess this ability. Staphopain A (ScpA) from Staphylococcus aureus (23), streptopain (SpeB) from Streptococcus pyogenes (42), 56K-protease from Serratia marcescens (43), and subtilisin from Bacillus subtilis (43) were capable of releasing kinin directly from human kininogens. The fact that ASP generated VL activity from both kininogens (Fig. 6) is in agreement with the kinin-releasing ability of these proteinases (23, 42, 43), with the exception of subtilisin, a serine protease, that releases kinin only from LK (43). ASP produced more VL activity from kininogens than ScpA, the proteinase that generates kinin most efficiently of these four listed enzymes (Fig. 6 and Ref. 23). Therefore, ASP would appear to be the most potent in vitro kinase generator of any bacterial protease studied to date. However, the result that ASP generated less kinin from human plasma than ScpA devoid of prekallikrein activation ability (Fig. 4 and Ref. 23), suggests a strong interference of plasma components, including protease inhibitors, in ASP kinin production when compared with ScpA.

The partial inhibition of ASP VL by diphenhydramine (Figs. 2B, inset, and 4) demonstrates the participation of histamine release from mast cells. A Vibrio mimicus metalloprotease and a house dust mite serine protease have been shown to cause histamine-dependent VL (44, 45). The mite protease and gingipain-R release anaphylatoxin (C3a and C5a)-like fragments from complement C3 and C5, respectively, which elicit histamine release from mast cells (45, 46); and C5a also induces neutrophil chemotaxis (46). Indeed, significant neutrophil accumulation was seen at ASP injection sites, but not DFP-treated ASP injection sites in guinea pigs (data not shown). Thus, anaphylatoxin-like molecule production could be a mechanism of H1 receptor-dependent VL by ASP. The histamine-induced VL terminates within a few minutes, and, once released, histamine is not restored in mast cells for at least several hours; hence, mast cells cannot continue histamine release, even though ASP continues to initiate anaphylatoxin generation. Conversely, because of the continuous supply of prekallikrein and kininogens to ASP injection sites from plasma due to VL, their local concentrations increase to plasma levels until the proteinase is completely inactivated; presumably by α2-macroglobulin, which is the most potent plasma protease inhibitor for bacterial proteases (47, 48), and is found to increase at VL sites (49).

These observations may explain the result that histamine was initially involved for only a short period, and afterward the kallikrein/kinin system contributed almost all to ASP-induced VL (Fig. 3). To induce histamine release, anaphylatoxins, when produced in the blood stream by ASP, must permeate to the extravascular space where mast cells reside. Due to dilution in blood, and diffusion in the extravascular space, the anaphylatoxins are probably unable to keep a concentration sufficient to elicit mast cell histamine release. Kinins generated in the blood stream can bind directly to B2 receptor on endothelial cells and cause BP reduction via vasodilation through the production of NO and prostacyclin (50), in addition to by VL. As shown by
the finding that the BP-lowering activity of ASP was completely inhibited by HOE140 (Fig. 8), it is clear that the kallikrein/kinin system plays a major role in the effect of ASP on blood vessels in contrast to the marginal contribution of the histamine pathway.

We have conclusively demonstrated that ASP, secreted from *A. sobria* at infection sites or in the circulation, is likely to produce kinin, possibly contributing to septic shock through its value of assessing contact system activation and factor V in systemic inflammation.

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