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Production of *Vibrio vulnificus* hemolysin in vivo and its pathogenic significance

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Abstract

Hemolyin/cytolysin (VvhA) is one of the representative exotoxins produced by *Vibrio vulnificus*. Cytotoxic mechanism of VvhA has been extensively studied. However, there have been controversies concerning the pathogenic significance since *vvhA*$^-$ mutant showed no LD_{50} change in mice. In this study, we investigated whether VvhA is expressed in vivo. When *V. vulnificus* was cultured in the presence of normal pooled human serum, substantial amount of VvhA was detected by ELISA and the transcription of *vvhA* was also evidently confirmed by RT-PCR and a transcriptional reporter assay. To investigate whether VvhA is expressed in vivo, mice were infected with *V. vulnificus* and bacterial RNAs were harvested from the mice. In vivo *vvhA* transcription was observed evidently by RT-PCR. We hereby propose that VvhA is substantially produced in vivo and would contribute to the pathogenesis of *V. vulnificus* septicemia.

Keywords: *Vibrio vulnificus*; Hemolysin/cytolysin (VvhA); Septicemia; Virulence; In vivo production; RT-PCR; ELISA; Cytotoxicity; Transcriptional reporter; Normal pooled serum

Vibrio vulnificus is a halophilic estuarine bacterium that causes fulminant septicemia, resulting in high mortality above 50% in patients who have underlying hepatic diseases [1–3]. Several bacterial components or characteristics have been suggested as the virulence factors of *V. vulnificus* through in vitro or in vivo experiments. Suggested virulence factors are as follows: an extracellular hemolysin/cytolysin [4–6] and a protease [7,8], a phospholipase A2 [9], a polysaccharide capsule [1,10–12], resistant to the serum bactericidal activity [13,14] and phagocytosis [15], and the iron assimilation system [16,17]. Among the exotoxins, hemolysin/cytolysin (VvhA) proved to be the most potent one. Intravenously administered VvhA kills mice at very low dosages [18]. The VvhA lyses red blood cells and shows cytolytic activity in cultured cell lines [4,18]. In animal models, locally and systemically administered VvhA reproduces the same clinical and pathological manifestations of the septicemia as caused by the administration of live bacteria [4–6,18,19]. However, there have been controversies concerning the pathogenic significance of VvhA. Wright and Morris [20] showed that inactivation of *vvh A* hemolysin gene did not affect the virulence of *V. vulnificus* and they suggested that VvhA has nothing to do with the virulence of *V. vulnificus*. On the other hand,

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biological effects of other proposed virulence factors reported to date are not sufficient to explain the fulminant process of the septicemia and the dermatologic manifestations such as hemorrhagic bullae formation or soft tissue necrosis. Our laboratory found that the VvhA directly causes hypotension and tachycardia in rats, and dilates rat thoracic aorta at far lower concentration than that is required for cytotoxicity [21,22]. We suppose that the VvhA should still play an important role in the pathogenesis of hypotensive shock though it is not a decisive virulence factor involved in the establishment of infection that could be tested by the mouse lethality model. All *V. vulnificus* strains tested have been shown to carry the gene (*vvhA*) [23,24]. From a standpoint of evolution, *V. vulnificus* should have maintained the gene because it was essential for survival. If VvhA is necessary for the in vivo growth of *V. vulnificus*, it should be produced when the bacterium comes into animal bodies. Pathogenic bacteria express virulence genes preferentially in hosts, which are essential for the establishment of infection while they turn off those genes needed solely for environmental survival [25,26]. Our most fundamental question asked in this study was whether VvhA is made in vivo to a critical level. This study shows that VvhA is substantially produced by the *V. vulnificus* cells growing in vivo.

Materials and methods

Bacterial strain and growth conditions. Vibrio vulnificus C7184 was kindly provided by James D. Oliver of North Carolina University at Charlotte. *V. vulnificus* MO6-24/O, capsule negative isogenic mutant CVD752 [27], and hemolysin negative isogenic mutant CVD707 [20] were provided by J. Glenn Morris of the University of Maryland. *V. vulnificus* strains were grown in 2.5% NaCl heart infusion (HI) medium at 37 °C.

Hemolysin (VvhA) assay. Purified VvhA was a kind gift from J.W. Park of Chonbuk National University, Korea [28]. Hemolytic activity in the culture supernatant was assayed as described by Shinoda et al. [29]. It was expressed as hemolytic unit (HU) that is the reciprocal of the maximal dilution showing 50% hemolysis.

Cell culture and cytotoxicity assay. Chinese hamster ovarian CHO-K1, bovine pulmonary artery endothelial CPAE cell lines, and peritoneal macrophages from CD-1 mice were used to evaluate cytotoxicity of VvhA. CHO-K1 cells were grown in F-12 medium supplemented with 10% FBS, penicillin, and streptomycin. CPAE cells were grown in Earl's salt minimal essential medium (EMEM) supplemented with 20% FBS, penicillin, and streptomycin. CHO-K1, CPAE, or macrophage cells were seeded in 96-well culture plates to 4×10^4/well and cultured at 37 °C and 5% CO_2. After washing with serum free media, the cells were treated with various concentrations of purified VvhA or culture supernatant for 1 h. Cytotoxicity of the VvhA was evaluated by the XTT assay kit (Roche Applied Science, Sandhofer Strasse, Germany). Cytotoxic activity of live bacteria was measured by LDH release from the cells using Cytotox 96 Non-Radioactive cytotoxic assay kit (Promega, Madison, WI) or trypan blue exclusion assay.

ELISA. We determined relative VvhA concentration in the culture supernatant by ELISA using an anti-VvhA monoclonal antibody. The anti-VvhA monoclonal antibody was elicited from Balb/c mice using purified VvhA as an antigen as described [21]. *V. vulnificus* C7184 was cultured in 2.5% NaCl heart infusion (HI) supplemented with or without 20% normal pooled human serum (NPS) and culture supernatants were collected by centrifugation. Flat-bottomed 96-well ELISA plates (Corning Laboratories, Corning, NY) were coated with culture supernatant and incubated at 4 °C overnight. After extensive washing with distilled water, the plates were incubated with a blocking buffer (5% fetal bovine serum, 0.05% Tween 20, 1 mM EDTA, and 0.5% BSA in phosphate-buffered saline) for 3 h to prevent non-specific interaction. Five hundred nanograms per milliliter of anti-VvhA monoclonal antibody was incubated for 2 h. After washing with distilled water, 50 μl of anti-mouse horseradish peroxidase conjugate (Sigma) diluted with the blocking buffer was added to each well, and the plates were incubated for one more hour at 37 °C and then developed with the 50 μl of substrate TMB (3,3′,5,5′-tetramethylbenzidine) solution (Sigma). The reaction was stopped by adding 50 μl of 1 N H_2SO_4 (Sigma) and the absorbance of each well was read by a microplate reader (Molecular Devices, Menlo Park, CA) at 450 nm.

RT-PCR. To evaluate transcriptional activity of *vvhA*, total RNA was extracted from the bacteria or liver from moribund mice infected with *V. vulnificus* with the TRIzol reagent (Invitrogen, Carlsbad, CA), and 3 μg of total RNA was reverse transcribed into cDNA. The *vvhA* gene transcription levels were compared by PCR using a sense primer (5′-GCTATTTCACCGCCGCTCAC-3′) and an antisense primer (5′-CCGCAGAGCCGTAAACCGAA-3′) [24].

β-Galactosidase assay. Vibrio vulnificus CMM2105 [28], the *vvhA*::*lacZ* transcriptional reporter, was grown in 2.5% NaCl HI broth overnight by shaking at 200 rpm and 37 °C. Overnight culture of the strain was inoculated in 100 ml fresh 2.5% NaCl HI broth or 2.5% NaCl HI broth supplemented with 20% NPS to the concentration of 5×10^6 CFU/ml. Cells were collected at appropriate time intervals by centrifugation at 13,000 rpm for 1 min using a microcentrifuge and stored at −20 °C. β-Galactosidase activity of the reporter strain was assayed as described [30]. All the assays were performed in triplicates.

In vivo vvhA transcription. Vibrio vulnificus C7184 cultured in 2.5% NaCl HI broth for 6 h at 37 °C with agitation was harvested by centrifugation. After washing twice with phosphate-buffered saline (PBS), the bacterial cells were resuspended with PBS to 5×10^7 CFU/ml and 0.2 ml of the bacterial suspension was intraperitoneally injected into mice. Bacterial RNAs were extracted from liver of nearly dying septicemic mice using a total RNA extraction kit (Qiagen, Hilden, Germany) and RT-PCR was done as described above. The liver was homogenized before the RNA extraction. The number of viable *V. vulnificus* harvested from liver was calculated by the pour plating method and, as a control, the same number of *V. vulnificus* cultured in vitro as described above was added to normal mouse liver and total RNA was extracted from each sample. Contamination of the RNA with chromosomal DNA fragments was thoroughly ruled out by a RNase free DNase treatment and the subsequent confirmation of PCR-negativity of the RNA preparation.

Results and discussion

Effect of vvhA inactivation on cytotoxicity

To investigate the effect of VvhA on bacteria-mediated cytotoxicity, we compared the cytotoxic activity of *V. vulnificus* parent strain MO6-24/O and hemolysin negative mutant CVD707 on mouse peritoneal macrophages. Phagocytes seem to be one of the major cellular targets that *V. vulnificus* encounters during the infection process. MO6-24/O strain was highly cytotoxic to murine peritoneal macrophages while CVD707 strain

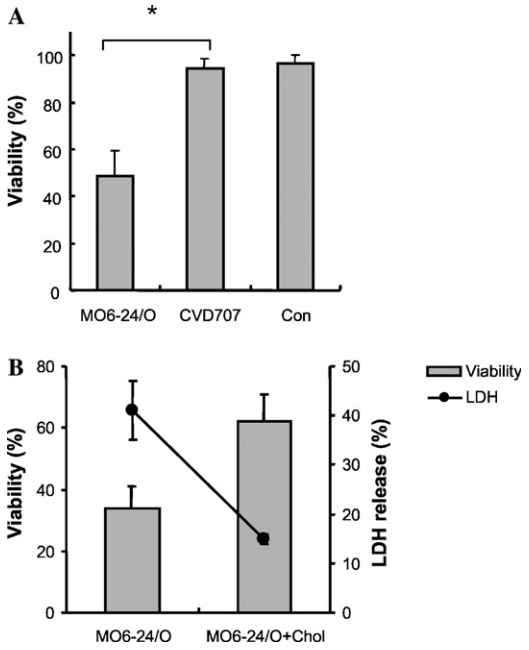

Fig. 1. Effect of *vvhA* inactivation on the cytotoxicity of *V. vulnificus* to mouse peritoneal macrophage (PEM). (A) Mouse peritoneal macrophages were obtained from CD-1 mice by thioglycolate enrichment and incubated with MO6-24/O and CVD707 at MOI (multiplicity of infection) 100 for 1 h. Viability of the PEMs was evaluated by trypan blue exclusion method. (B) PEMs were incubated with *V. vulnificus* MO6-24/O in the presence or absence of cholesterol, and the viability of the cells was determined by trypan blue exclusion test (column) and LDH release (line) from the PEMs. Data represent means ± SEM. Asterisk indicates statistically significant difference from the control group (*$p < 0.01$).

showed less cytotoxicity (Fig. 1A). In addition, cholesterol, a well-known substance that blocks VvhA-mediated hemolysis [4,22,31], effectively inhibited cytotoxicity of the wild type MO6-24/O on murine peritoneal macrophage (Fig. 1B). This result strongly suggests, in spite of the previous report of Wright and Morris [20] showing that VvhA-negative *V. vulnificus* mutant showed no difference in the LD_{50} from the wild type, that VvhA should play an important role for live *V. vulnificus* in eliciting cytotoxicity in vivo.

In vitro VvhA activity in the presence of NPS

The fundamental question of this study was how toxic was the VvhA under in vivo situation. This question had been provoked by the report of Wright and Morris [20], denoting that VvhA-negative *V. vulnificus* mutant showed no difference in the LD_{50} from the wild type. To investigate the pathogenic role of VvhA in vivo, we observed the biological activity of VvhA in the presence of human serum, which in part reconstitutes in vivo situation. First, we determined the effect of 20% NPS on hemolysis and cytotoxicity caused by purified VvhA. As shown in Fig. 2, 20% NPS significantly inhibited hemolytic and cytotoxic activities of VvhA at 5 HU/ml as well as at 1.25 HU/ml. This suggests that VvhA could be inactivated in vivo situation. This result suggests that the activity of VvhA, even if it was produced in the bloodstream, might be compromised by serum constituents such as cholesterol in vivo. Second, we detected the production of VvhA from the culture supernatant when the bacterium was cultured in the presence of 20% NPS. Though the growth of *V. vulnificus* was not significantly affected by the presence of NPS in medium (Fig. 3A), surprisingly no hemolytic activity was detected in the culture supernatant throughout 48 h of culture (Fig. 3B). There could have been two possible hypotheses for the absence of hemolytic activity in the cultures supplemented with 20% NPS. One is that NPS inhibited the VvhA production by *V. vulnificus*. The other is that NPS inactivated VvhA that was produced by the pathogen, even in the presence of serum. To prove the hypotheses, the following experiments were carried out.

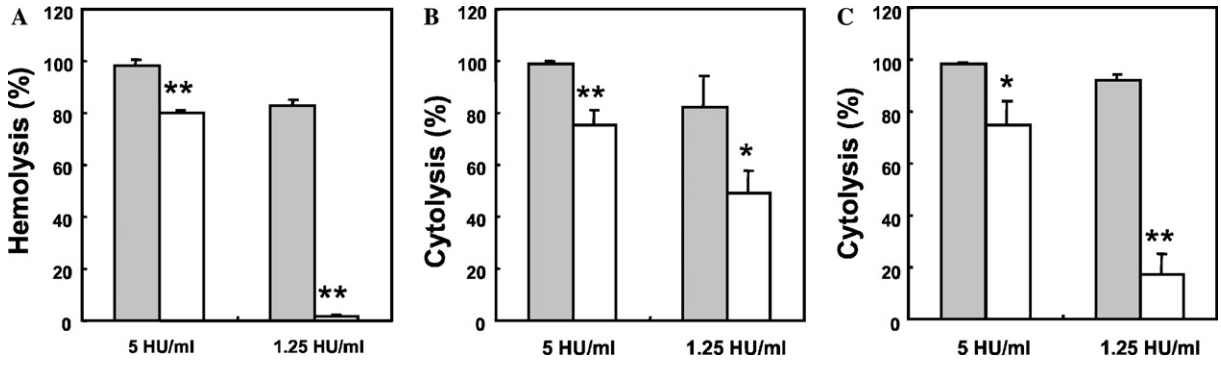

Fig. 2. Protective effect of the NPS on the hemolytic and cytotoxic activities of the VvhA. (A) Purified VvhA (5 or 1.25 HU/ml) was incubated with 1% human RBC solution supplemented with (white bar) or without 20% NPS (gray bar) for 1 h. Released hemoglobin in the supernatant was measured at 540 nm. Percent hemolysis is presented as mean ± SEM. Purified VvhA (5 or 1.25 HU/ml) was added to culture of CHO-K1 (B) and CPAE ells (C) in the presence (white bar) or absence of 20% NPS (gray bar) and incubated for 1 h. Viability of the cell lines was calculated by the XTT assay. Percent cytolysis is presented as mean ± SE. Asterisk indicates statistically significant difference from the control. (*$p < 0.05$, **$p < 0.005$).

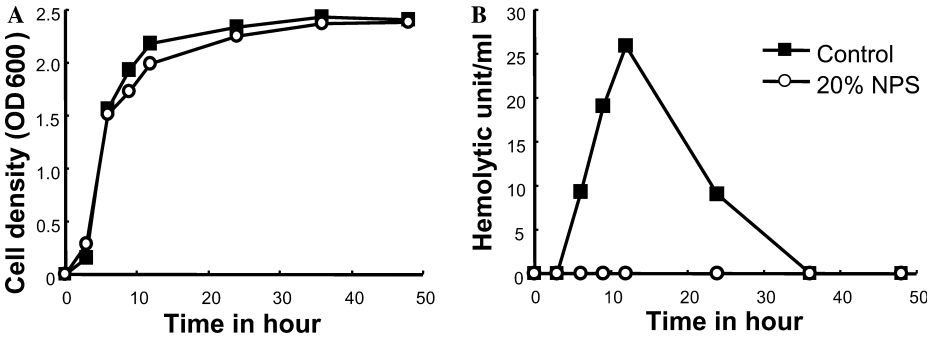

Fig. 3. Growth and VvhA production of *V. vulnificus* C7184 inoculated in 2.5% NaCl HI broth supplemented with or without 20% NPS. (A) Bacterial growth was not significantly affected by the presence of NPS in the medium. (B) When the bacterium was cultured in the medium supplemented with 20% NPS, no hemolytic activity could be detected in the supernatant.

Evaluation of in vitro VvhA expression in the presence of 20%

We tried to detect immunologically reactive VvhA in the culture supernatants containing 20% NPS. The VvhA was measured by ELISA using an anti-VvhA monoclonal antibody. The monoclonal antibody neutralized the hemolytic activity of VvhA and was proved to react specifically with the VvhA band by immunoblotting (data not shown). As shown in the Fig. 4A, substantial amount of VvhA was detected in the 2.5% NaCl HI broth containing 20% NPS though the peak level was approximately half of that detected in control culture without serum. Transcriptional activation of *vvhA* gene in the presence of 20% NPS in vitro was also assayed by the β-galactosidase assay (Fig. 4B) and RT-PCR (Fig. 4C). CMM2105, a chromosomal *vvhA::lacZ* reporter strain, was cultured in 2.5% HI broth supplemented with or without 20% NPS. As shown in Fig. 4B, transcriptional activity of *vvhA* significantly decreased in the presence of 20% NPS. *V. vulnificus* C7184 was inoculated in 2.5% NaCl HI supplemented with or without 20% NPS. As shown in Fig. 4C, RT-PCR result correlated with ELISA and the transcriptional reporter assay results. The *vvhA* transcription was evident, though less *vvhA* mRNA was produced, when *V. vulnificus* was cultured in the presence of NPS. These results suggest that the lack of hemolytic activity in the culture supplemented with human serum is not because the VvhA was not produced, but produced VvhA should have been inactivated by the serum as observed in the above cytotoxicity experiments.

In vivo expression of vvhA

To investigate whether VvhA is expressed in vivo, ca. 10^7 CFUs of *V. vulnificus* was intraperitoneally infected. Bacterial RNA was harvested 6 h after the infection when the mice were nearly dying, and the expression of *vvhA* gene was evaluated by RT-PCR. As shown in Fig. 5, expression of *vvhA* was observed in the bacterial cells infecting host livers though the level was relatively lower than that in the same number of bacteria cultured in vitro in a nutritious medium. This result obviously proved that that the *vvhA* gene is expressed in vivo.

Significance of vvhA expression in vivo

We previously found that VvhA dilates the rat aorta at far less concentrations in comparison with in vitro cytotoxic concentrations [21]. In this study, we determined the effective dosages of purified VvhA for cytotoxicity, vasodilatation, and lethality to mice. To determine the cytotoxic dose 50 (CD_{50}), CHO-K1 and CPAE cell lines were incubated with various concentrations of VvhA for 1 h, and cytotoxicity of VvhA was measured by the XTT assay. Based on the dose–response curves, we calculated CD_{50} in each cell lines. CD_{50}'s for CHO-K1 and CPAE were 0.55 and 0.37 HU/ml, respectively. We also determined IC_{50} for vasodilator activity of VvhA in phenylephrine-precontracted rat aortic ring as described earlier [22]. It was 0.0055 HU/ml. To determine LD_{50} of VvhA, 8-week-old Balb/c mice were intravenously injected with various concentrations of VvhA and observed for 24 h. LD_{50} of the VvhA calculated by Reed–Muench method [32] was 5.6 HU/mouse (Table 1). According to these results, purified VvhA has a potent vasodilator activity at the concentration of about 2 log scale lower than that is required for cytotoxicity to cultured cell line. Gray and Kreger [4–6] showed that *V. vulnificus* VvhA were much more lethal for mice by intravenous administration than by intraperitoneal injection, the difference for the LD_{50} by the two routes was ca. 830-fold, and the VvhA caused extensive extracellular edema and increased vascular permeability at the injection sites. These phenomena strongly suggest that the cellular targets of VvhA needed for the establishment of fatal hypotensive shock should be vascular cells. Pathophysiologically significant vasodilatation and consequent hypotension might occur much prior to VvhA-induced overt damage of endothelial cells and surrounding tissues. Since VvhA shows

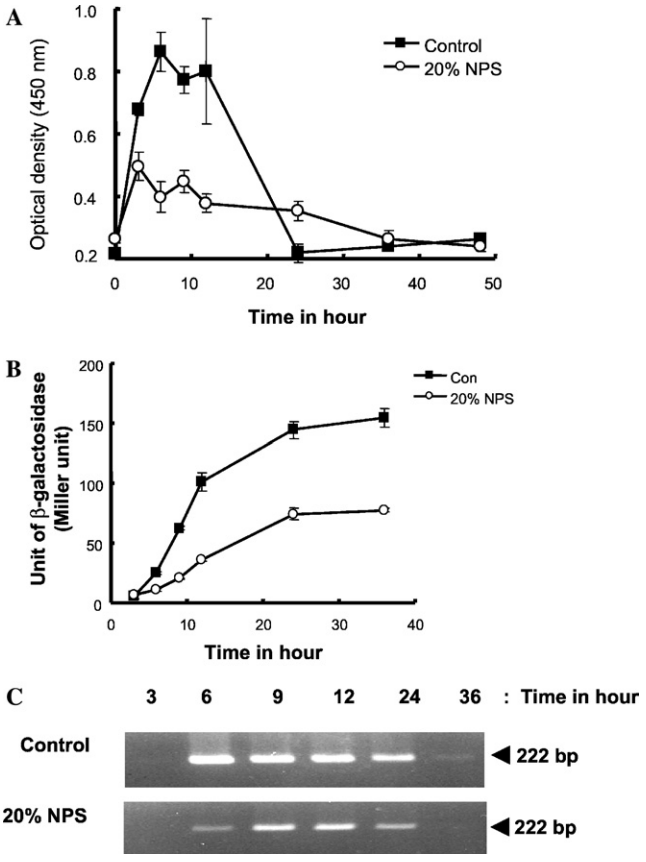

Fig. 4. VvhA production by *V. vulnificus* C7184 inoculated in 2.5% NaCl HI broth supplemented with or without 20% NPS. (A) Immunologically detectable VvhA was observed in the culture supernatant by ELISA. Significant amount of VvhA could be detected in the 20% NPS HI broth though the peak level was about half of that detected in control culture without NPS. Each point in the curve represents mean ± SE. (B) Effect of human serum on the *vvhA* transcriptional activation. Aliquots of overnight culture of CMM2105, a *vvhA::lacZ* transcriptional reporter strain of MO6-24/O with *lacZ* deletion, were incubated with 2.5% NaCl HI broth supplemented with or without 20% NPS. Transcriptional activity was determined by the β-galactosidase assay. (C) Expression of the VvhA in *V. vulnificus* C7184 in 20% NPS supplemented media. One microgram of total RNA was reverse transcribed into cDNA and used as the template for the PCR.

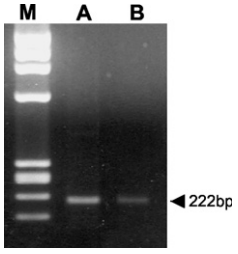

Fig. 5. Expression of the *vvhA* in *V. vulnificus* C7184 in vivo. Approximately 10^7 CFUs of the bacterium was infected to Balb/c mice intraperitoneally. Bacteria were harvested from the liver 6 h after infection when the mouse suffered from septicemia and was nearly dying. cDNA was synthesized from 3 μg of the total RNA extracted from the homogenized liver tissue and used as the template for the PCR. Lane M, *Hae* III-ϕ 174 DNA marker; lane A, RT-PCR result using RNA extracted from bacteria grown in vitro; and lane B, RT-PCR result using RNA extracted from bacteria grown in vivo.

Table 1
Dosage of VvhA required for biological activities

Biological activity	Descriptions	Unit
Cytotoxicity	CD_{50}[a] in CHO-K1 cell line	0.55 HU/ml
	CD_{50} in CPAE cell line	0.37 HU/ml
Vasodilatation	IC_{50}[b]	0.0055 HU/ml
Lethality to mice	LD_{50}[c]	5.60 HU/mouse

[a] Cytotoxic dose 50: the cell lines were incubated with various concentrations of VvhA for 1 h and viability was measured by XTT assay. From the dose–response curves, CD_{50}s were calculated.

[b] Inhibitory concentration 50: the rat thoracic aortic rings (AOs) were precontracted with 10^{-6} M phenylephrine and vasodilatory effect of VvhA was tested. IC_{50} was calculated from the typical dose–response curves. Eight AOs were tested in this experiment.

[c] Lethal dose 50: the VvhA was injected intravenously and observed for 24 h. LD_{50} was calculated by the Reed–Muench method.

high affinity to cholesterol [4,31], locally produced VvhA should first bind to cell membranes nearby, and spilled-over VvhA seems to reach circulation triggering subsequently systemic vasodilatation and hypotensive shock. Early diagnosis and extensive debridement of skin lesions are essential to save life from *V. vulnificus* septicemia [33]. High concentration of *V. vulnificus* is discovered in bulla fluid and necrotic tissue, even when blood cultures are negative. This study strongly suggests that *V. vulnificus* cells in the local necrotic tissue produce a certain critical level of VvhA that can cause local tissue damage and systemic hypotension. In conclusion, we suggest that (1) VvhA is produced in vivo, (2) but the amount is relatively smaller than in vitro production, and (3) such small amount might be sufficient for causing biological effects such as hypotension.

Acknowledgments

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