Cytotoxicity of Vibrio vulnificus cytolysin on pulmonary endothelial cells

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Abstract

The cytolysin produced by V. vulnificus has been known to cause lethality by increasing pulmonary vascular permeability in mice. In the present study, its cytotoxic mechanism on CPAE cell, a cell line of pulmonary endothelial cell, has been investigated. The cytolysin rapidly bound on CPAE cells and killed approximately 80% of the cells at 1.0 HU as determined by trypan blue exclusion test. The death of CPAE cells was associated with the formation of transmembrane pore evidenced by rapid flow of monovalent ions in patch clamp of CPAE cell membrane. The cytolysin decreased cellular ATP levels to less than 30% of control at 0.25 HU. The incubation of cytolysin in the presence of cholesterol completely inhibited its cytotoxic effect on CPAE cells, suggesting that cholesterol on CPAE cell membrane is the probable binding site for cytolysin. These results suggest that the death of CPAE cell induced by cytolysin is due to the depletion of cellular ATP levels by the formation of small transmembrane pores, which are permeable to monovalent ions, but not to LDH.

Keywords: *Vibrio vulnificus*, cytolysin, pulmonary endothelial cell

Introduction

Vibrio vulnificus is an estaurine bacterium that is recognized as an etiological agent of septicemia and serious wound infection in humans who are immunocompromised or have underlying diseases such as liver cirrhosis or hemochromatosis (Hollis *et al*., 1976; Blake *et al*., 1980; Park *et al*., 1991). Kreger and Lockwood (1981) demonstrated that the cytolysin present in culture medium of *V. vulnificus* showed hemolytic activity and cytotoxicity for mammalian cells in culture, and acted as a vascular permeability factor. Even sub-microgram amount of the cytolysin is fatal to mice when injected intravenously (Gray and Kreger, 1985; Park *et al*., 1996). The extensive tissue damage which occurred in mice after subcutaneous injection of the cytolysin, and was very simillar to that observed during *V. vulnificus* wound infections (Gray and Kreger, 1989). These reports suggest that the cytolysin is the major factor in the pathogenesis of *V. vulnificus* infections. Although several lines of investigations on the the action of the cytolysin against mammalian cells such as mast cells (Yamanaka *et al*., 1990) and erythrocytes (Kim *et al*., 1993; Park. *et al*., 1994; Chae *et al*., 1996) has been carried out, the role of the cytolysin in the pathogenesis of the disease is not well known.

Recently, it has been known that *V.vulnificus* cytolysin have lethal activity primarily by increasing vascular permeability and neutrophil sequestration in the lung of mice (Park *et al*., 1996), and causes hypotension in rats (Kook *et al*., 1996). Rinaldo and Rogers (1986) indicated that septicemia and associated alterations of pulmonary endothelial function such as increased permeability of the pulmonary microvasculature by bacterial toxins are important in the pathogenesis of the acute respiratory distress syndrome.

This paper shows that *V.vulnificus* cytolysin has a direct cytotoxic effect on CPAE cells, a pulmonary endothelial cell line, which may be the target cells of cytolysin *in vivo*.

Materials and Methods

Bacterial strain and culture

A virulent strain of *V. vulnificus* E4125 was kindly supplied by Dr. M. H. Kothary (Department of Microbiology, Virulence Assessment Branch, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington D.C.). The strain was cultured in the heart infusion diffusate broth (Gibco, Gaithersburg, U.S.A.) at 37°C for 4 h as described by Kreger *et al*. (1988).

CPAE cell culture

The pulmonary endothelial cell line, CPAE (ATCC CCL 209) was obtained from Korea Cell Bank (College of Medicine, Seoul National University, Korea). CPAE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 units/ml), streptomycin (0.1 mg/ml) and amphotericin B (0.25 μg/ml) in a humidified atmosphere of 5% CO_2. CPAE cells were used after trypsinization.

Preparation of V. vulnificus cytolysin

The cytolysin was purified to homogeneity from the culture supernatant by ammonium sulfate fractionation, calcium phosphate gel adsorption, quartenary methylamine anion-exchange chromatography and octyl-Sepharose CL-4B chromatography as described by Kim *et al*. (1992).

Assay of cell viability

Cell viability was determined by Trypan blue dye exclusion method (Rosengard and Cochrane, 1983).

Assay of hemolytic activity

The hemolytic activity of cytolysin against mouse erythrocytes was determined by the method of Bernheimer and Schwarz (1963). The cytolysin was diluted with phosphate-buffered saline (PBS) (67 mM Na_2HPO_4, 77 mM NaCl, pH 7.4) containing 1 mg of bovine serum albumin (BSA) per ml. One milliliter of cytolysin was mixed with the same volume of 0.7% mouse erythrocyte suspensions in PBS-BSA. After incubation at 37°C for 30 min and brief centrifugation, absorbance of hemogolobin in the supernatant was measured. One hemolytic unit (HU) is defined as that amount which liberates half of the hemoglobin under the above conditions.

Release of lactate dehydrogenase (LDH)

The release of LDH was used as a marker for overt cytolysis. Enzymatic activity of LDH in the culture medium was determined by the method of Whalefeld (1983). Twenty microliters of the supernatant after brief centrifugation was added to 20 μl of 172 mM β-NAD and 460 μl of Tris/L-lactate (112 mM Tris, 56 mM L-lactate, 170 mM KCl, pH 9.3) and incubated. The increase of absorbance for 1 min at room temperature was monitored at 340 nm. Enzyme release was expressed as the percentage of total enzymatic activity determined by lysing cells with 5 μl of 5% Triton X-100.

Measurement of ATP

To measure cellular ATP, CPAE cells were lysed with 5% trichloroacetic acid. After brief centrifutgation, ATP in the supenatant was determined by the chemiluminescence method using luciferin-luciferase (Ludin, 1978). Twenty microliters of the supernatant was added to 80 μl of 100 mM glycine buffer (pH 12.6) and 100 μl of luciferin-luciferase solution, and the chemiluminescences were read by chemiluminometer (Lumat, LB9501). A standard ATP was prepared just before use.

Current recording

Gigaseals were formed with Sylgard-coated pipette (borosilicate, Kimble) with 4 to 5 megaohms resistance and single-channel currents were recorded by use of the inside-out configuration of the patch clamp method described by Hamill *et al*. (1981). Channel currents were recorded with an Axo-patch 1D patch clamp amplifier (Axon) and stored on video tapes via digital recorder (PCM-501ES, SONY) for later computer analysis. A pClamp software (Axon) was used for data acquisition and analysis. All experiments were carried out at 22 ± 2°C The standard bath and pipette solutions contained 140 mM KCl, 2m M $MgCl_2$, 5 mM EGTA and 10 mM HEPES (pH 7.2).

Results and Discussion

Many studies have reported about the effects of endotoxin on the pulmonary microvasculature (Brigham and Meyrick, 1986). Recently, Park *et al*. (1996) demonstrated that *V. vulnificus* cytolysin caused lethality primarily by increasing vascular permeability and neutrophil sequestration in the lung of mice. It has been known that *Escherichia coli* hemolysin increases the permeability of pulmonary artery endothelial cell monolayers (Suttorp, *et al*., 1990), and the stimulation of polymorphonuclear leukocyte adhesion to endothelial cells by bacterial exotoxins may be relevant in patient with severe local or systemic bacterial infections (Krüll *et al*., 1996). However, alterations of endothelial cell functions by bacterial exotoxins have received little attention.

In order to determine the binding activity of cytolysin, CPAE cells were incubated with cytolysin at 4°C. After brief centrifugation, the remaining hemolytic activity of unbound cytolysin in the supernatant was determined (Figure 1). The binding of cytolysin to CPAE cells was so rapid that approximately 50% of 1 HU of cytolysin was bound to CPAE cells within 1 min. This result was similar to that of erythrocytes (Kim *et al*., 1993). Viability test for CPAE cells was carried out to discern whether the bound cytolysin induced death of the cells. Cytolysin killed CPAE cells in a dose-dependent manner, approximately 80% of the cells being dead by treatment with 1.0 HU of cytolysin (Figure 2). These results suggest that the cytolysin can directly induce the death of CPAE cells after binding on the cells.

Bhakdi *et al*. (1986) proposed that generation of trans-membrane pores is the mechanism underlying the action of pore-forming exotoxins on target cells. Kim *et al*. (1993) demonstrated that the cytotolysis of erythrocyte induced by *V. vulnificus* cytolysin is due to the formation of pores on cell membrane through the three steps; specific binding of cytolysin to the lipid bilayer, formation of small pores by the oligomerization of the cytolysin, and osmotic lysis. In order to determine whether the death of CPAE cells is accompanied by the pore formation, the cells were incubated with the cytolysin and single-channel currents were recorded by use of the inside-out configuration of the patch clamp method (Figure 3). The formation of transmembrane pores by cytolysin was evidenced by rapid flow of monovalent ions (K^+ or Cl^-) in the patch clamp
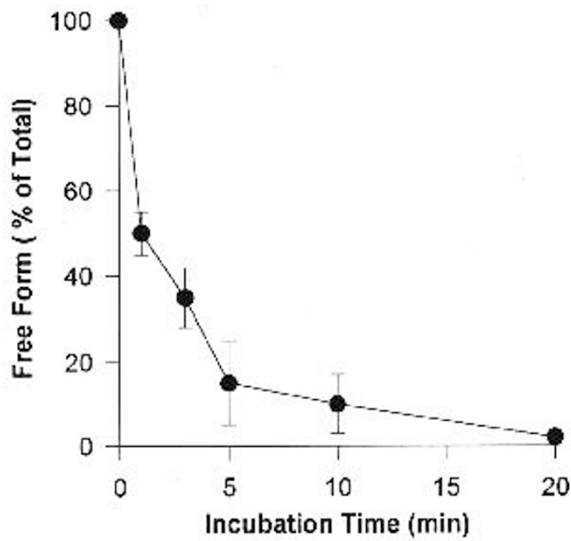
Figure 1. Binding of *V. vulnificus* cytolysin to CPAE cells. CPAE cells (1.3×10^5 cells/ml) were incubated with 1 HU of the cytolysin in a total volume of 1 ml of PBS-BSA at 4°C. After brief centrifugation, the residual cytolysin in supernatant at the given times was determined by its hemolytic activity as described in "Materials and Methods".The plot dipicts the mean ± SD obtained from four separate experiments.
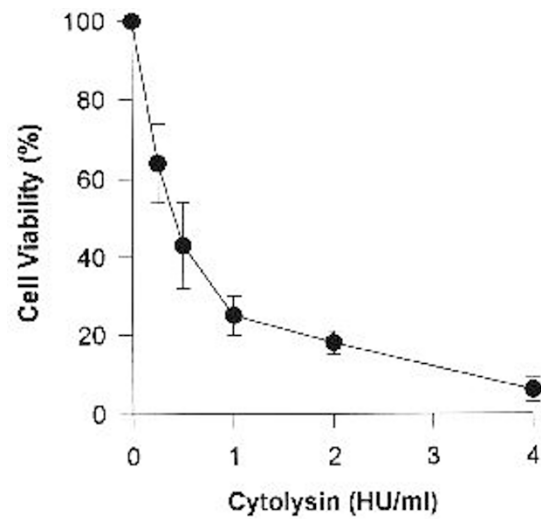
Figure 2. Effect of *V. vulnificus* cytolysin on the viability of CPAE cells. CPAE cells (1×10^6 cells/ml) were treated with various concentrations of the cytolysin in a total volume of 0.3 ml of PBS-BSA at 37°C for 30 min. Cell viability was determined by the ability to exclude trypan blue. Each value denotes the mean ± SD obtained from four separate experiments.
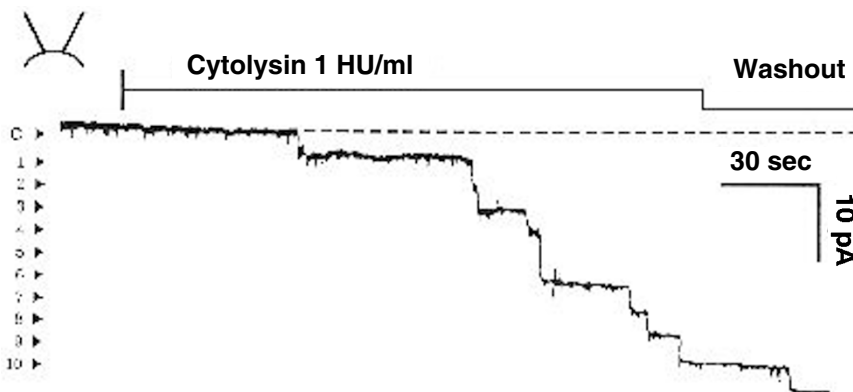

Figure 3. Effect of intracellularly applied *V. vulnificus* cytolysin on single channel currents in inside-out patches of CPAE cells. The bath and pipette solutions contained 140 mM KCl symmetrically, and the membrane potential was held at -40 mV. Cytolysin was added to bath solution after excision of patches. The filter for the tracing reproduction was set at 300 Hz. The number beside the current trace indicate the number to formed pores with C being the closed state.
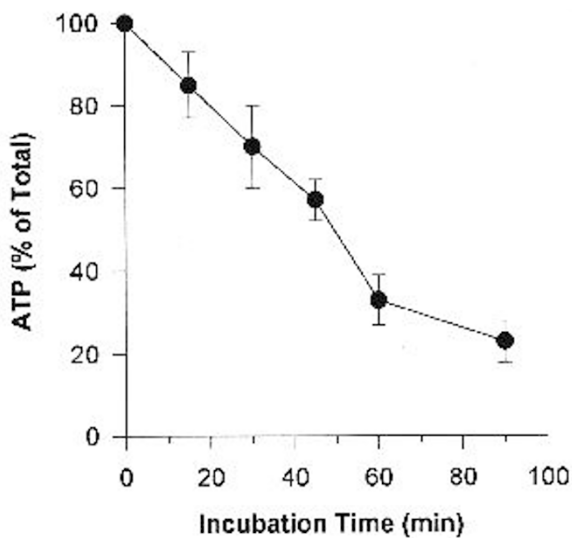
of CPAE cell membrane. To investigate the relationship between pore formation of cytolysin and cytolysis, the releases of LDH from the cells were determined (Figure 4). Erythrocyte LDH was released by the cytolysin but its release was completely prevented by raffinose, an osmotic protectant. Whereas, LDH of CPAE cells was not released by the cytolysin treatment even at the absence of raffinose without affecting cellular LDH level (data not shown). The result indicates that CPAE cells are not ruptured by the cytolysin-induced pore formation, being resistant to osmotic lysis in contrast to erythrocytes.

Cellular ATP level was measured, because the depletion of cellular ATP level is known as a hallmark of the deterioration of cell metabolism. As shown in Figure 5, cellular ATP was continuously depleted as a function of the incubation time at 0.25 HU of cytolysin, and approxi-

mately70% of total ATP was depleted within 60 min. When CPAE cells were also incubated with various concentrations of cytolysin, cellular ATP levels were decreased in a dose-dependent manner (Figure 6). Kim *et al*. (1993) demonst-rated that the pore formation on erythrocyte by *V. vulnificus* cytolysin is mediated by cholesterol on plasma membrane. Thus, to determine effect of cholesterol on ATP depletion of CPAE cells by cytolysin, cytolysin was incubated with cell suspensions containing cholesterol (1 μg/ml) (Figure 6). There was no leakage of ATP from CPAE cells into medium by the cytolysin (data not shown). The ATP depleting activity of the cytolysin on CPAE cells was completely inactivated by cholesterol. The redution of cellular ATP levels observed in this investigation was thought to be due to either the increase of ATP degradation or the decrease of ATP synthesis by mitochondrial
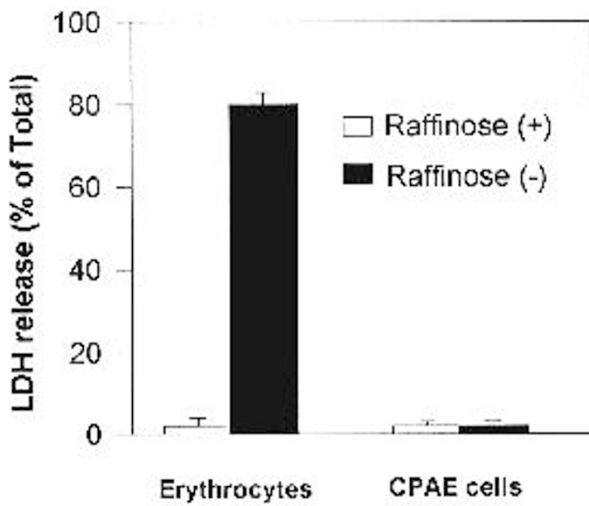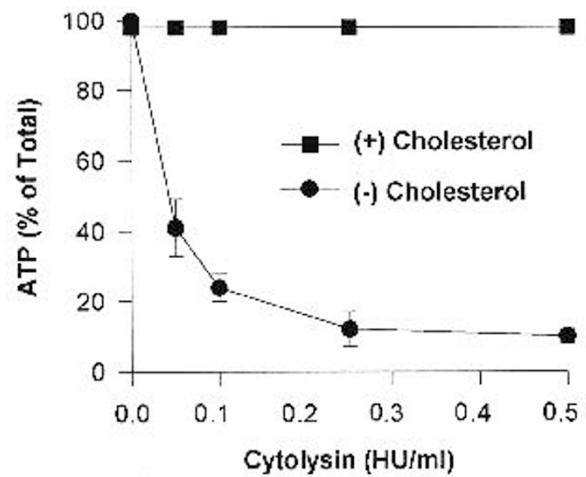
Figure 4. Effects of *V. vulnificus* cytolysin on LDH releases from mouse erythrocytes and CPAE cells. Mouse erythrocytes (0.35 %) and CPAE cells (1 × 10^6 cells/ml) were incubated with 1 HU of cytolsin in a total volume of 1 ml of PBS-BSA with or without 50 mM raffinose at 37°C for 30 min .The released LDH was determined as described in "Materials and Methods". Each value denotes the mean ± SD obtained from four separate experiments.

Figure 6. Effect of cholesterol on depletion of cellular ATP by *V. vulnificus* cytolysin. CPAE cells (1 × 10^6 cells/ml) were incubated with various concentrations of the cytolsin in a total volume of 1 ml of PBS-BSA with or without cholesterol (1 μg) at 37°C for 60 min .The cellular ATP content was determined as described in "Materials and Methods". Each value denotes the mean ± SD obtained from four separate experiments.

Figure 5. The time course of depletion of cellular ATP by *V. vulnificus* cytolysin. CPAE cells (1 × 10^6 cells/ml) were incubated with 0.25 HU of the cytolsin in a total volume of 1 ml of PBS-BSA at 37°C The cellular ATP content at the given times was determined as described in "Materials and Methods". Each value denotes the mean ± SD obtained from four separate experiments.

damage. Walev *et al*. (1993) demonstrated that the depletion of cellular ATP in keratinocytes by staphylococcal alpha-toxin was accompanied to the decrease of oxygen consumption, which was interpreted to reflect the breakdown of mitochondrial respiration.These results indicate that the toxic effect of *V. vulnificus* cytolysin on

CPAE cells may be initiated by binding of the cytolysin to cholesterol on cell membrane, and that the death of the cell is due to the pore formation.

The results of this study suggest that the cell death of CPAE cell induced by *V. vulnificus* cytolysin is due to the depletion of cellular ATP by the formation of small transmembrane pores, which are permeable to monovalent ions, but not to LDH.

Acknowledgement

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