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Vibrio vulnificus VvpE Stimulates IL-1β Production by the Hypomethylation of the IL-1β Promoter and NF-κB Activation via Lipid Raft–Dependent ANXA2 Recruitment and Reactive Oxygen Species Signaling in Intestinal Epithelial Cells

Sei-Jung Lee,*† Young Hyun Jung,*,† Eun Ju Song,*,† Kyung Ku Jang,‡ Sang Ho Choi,‡ and Ho Jae Han*,†

An inflammatory response is a hallmark of necrosis evoked by bacterial pathogens. Vibrio vulnificus, VvpE, is an elastase that is responsible for tissue necrosis and inflammation; however, the molecular mechanism by which it regulates host cell death has not been characterized. In the present study, we investigate the cellular mechanism of VvpE with regard to host cell death and the inflammatory response of human intestinal epithelial (INT-407) cells. The recombinant protein (r)VvpE (50 pg/ml) caused cytotoxicity mainly via necrosis coupled with IL-1β production. The necrotic cell death induced by rVvpE is highly susceptible to the knockdown of annexin A (ANXA)2 and the sequestration of membrane cholesterol. We found that rVvpE induces the recruitment of NADPH oxidase 2 and neutrophil cytosolic factor 1 into membrane lipid rafts coupled with ANXA2 to facilitate the production of reactive oxygen species (ROS). The bacterial signaling of rVvpE through ROS production is uniquely mediated by the phosphorylation of redox-sensitive transcription factor NF-κB. The silencing of NF-κB inhibited IL-1β production during necrosis. rVvpE induced hypomethylation and region-specific transcriptional occupancy by NF-κB in the promoter and has the ability to induce pyroptosis via NOD-, LRR-, and pyrin domain–containing 3 inflammasome. In a mouse model of V. vulnificus infection, the mutation of the vvpE gene from V. vulnificus negated the proinflammatory responses and maintained the physiological levels of the proliferation and migration of enterocytes. These results demonstrate that VvpE induces the hypomethylation of the IL-1β promoter and the transcriptional regulation of NF-κB through lipid raft–dependent ANXA2 recruitment and ROS signaling to promote IL-1β production in intestinal epithelial cells. The Journal of Immunology, 2015, 195: 2282–2293.

The most prominent feature of host–pathogen interaction is the elicitation of a diverse array of host-protective and stress responses, such as cell death, proliferation, and inflammation (1). Specifically, the host cell death response caused as a consequence of a bacterial infection results in various modes of cell death, including apoptosis, necrosis, and pyroptosis. This process eventually disturbs the fine balance between host innate defense barriers, which may be a key element in the development of certain diseases (2). The anaerobic Gram-negative bacterium Vibrio vulnificus is a rod-shaped, curved, and motile pathogen with a single polar flagellum that causes serious and often fatal infections in humans. V. vulnificus infection is characterized by a high fatality rate of 70% in South Korea. It primarily affects people who are immunocompromised or have underlying diseases such as liver cirrhosis (3, 4). The most prominent aspect of V. vulnificus pathogenesis is its ability to infect a host via the gastrointestinal tract, after which it rapidly spreads from the small intestine to the blood stream (5). Although most of the virulence effects of V. vulnificus are known to derive from secreted toxins encoded by cytolytic pore-forming hemolysin (VvhA) (5) and multifunctional autoprocessing repeats in the toxin (MARTXVv) (5, 6), several other secreted and cell-associated factors of V. vulnificus have also been proposed as potential virulence determinants that are also involved in the pathogenesis of V. vulnificus. A 45-kDa elastase designated as V. vulnificus VvpE is considered to be another possible virulence factor of V. vulnificus (7, 8). Purified VvpE was proven to be lethal to mice and to induce hemorrhagic damage and dermonecrosis (8–10). Although previous research has raised some serious doubts about whether this enzyme is not a major virulence factor involved in mouse lethality (11), VvpE was shown to cause tissue necrosis and increased vascular permeability that are necessary for the invasion of this bacterium (7, 12). Additionally, VvpE has the ability to regulate the activity of host cell death–related enzymes (13) and is essential for bacterial swimming and survival to promote the virulence effect of V. vulnificus (14). To date, however, the underlying

*Department of Veterinary Physiology, College of Veterinary Medicine, Research Institute for Veterinary Science, Seoul National University, Seoul 151-741, South Korea; †Brain Korea 21 Program for Leading Universities and Students (BK21 PLUS) Creative Veterinary Research Center, Seoul National University, Seoul 151-741, South Korea; and ‡Department of Agricultural Biotechnology, National Research Laboratory of Molecular Microbiology and Toxicology, and Center for Food Safety and Toxicology, Seoul National University, Seoul 151-921, South Korea.

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Address correspondence and reprint requests to Prof. Ho Jae Han, Department of Veterinary Physiology, College of Veterinary Medicine, Seoul National University, Gwanak-ro, Gwanak-gu, Seoul 151-742, South Korea. E-mail address: hjhan@snu.ac.kr

Abbreviations used in this article: ANXA, annexin A; S-Aza, 5-aza-2′-deoxycytidine; ChIP, chromatin immunoprecipitation; CM-H2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; CTB, cholera toxin subunit B; e.g., intragastrically; L.B., Luria–Bertani; MCD, methyl-β-cyclodextrin; MOI, multiplicity of infection; MSP, methyl-specific PCR; NAC, N-acetyl-cysteine; NCF1, neutrophil cytosolic factor 1; NLRP3, NOD-, LRR-, and pyrin domain–containing 3 inflammasomes; NOX, NADPH oxidase; PI, propidium iodide; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; rVvpE, recombinant protein VvpE; siRNA, small interfering RNA; WT, wild-type.

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cellular mechanisms of VvpE with regard to host cell death and the inflammatory response involved in this process remain largely unknown.

Whereas necrosis is associated with caspase-independent inflammation accompanied by nuclear swelling and the release of cellular contents, pyroptosis has been characterized as a programmed cell death coordinated by inflammasome-mediated caspase-1 activation that results in maturation of the proinflammatory cytokines IL-1β and IL-18 (2, 15). Pyroptosis is crucial for controlling microbial infections and can be regulated by reactive oxygen species (ROS) production, potassium efflux, or lysosomal damage (2, 15, 16). Epigenetic control of gene expression during a pathogenic bacterial infection is another important element in the regulation of host cell death. A recent report showed that many enteric bacterial pathogens, such as Salmonella typhimurium (17, 18), Helicobacter pylori (19), and the enteropathogenic Escherichia coli (EPEC) (20), affect a diverse set of epigenetic factors such as DNA methylation and histone modification to regulate the selective activation or silencing of specific host genes (21). Thus, studies regarding the process leading to pathogen-induced cell death are likely to be critical to uncovering the mechanisms of pathogenesis. However, there are no previous reports related to the molecular mechanisms of VvpE action, which drive the transcriptional and epigenetic regulation of the inflammatory process during the necrosis of intestinal epithelial cells. In this study, therefore, we investigate the VvpE signaling pathway that leads to host cell death coupled with the inflammatory response and evaluate the functional importance of VvpE in a mouse model of V. vulnificus infection.

Materials and Methods

Chemicals

FBS was purchased from BioWhittaker (Walkersville, MD). The following Abs were purchased: NADPH oxidase (NOX2, annexin A (ANXA2), and ANXA4 Ab (BD Biosciences, Franklin Lakes, NJ); neutrophil cytosolic factor 1 (NCF1) Ab (LifeSpan Biosciences, Seattle, WA); p-NF-κB p65, NF-κBp65, IL-1βp17, caspase-1p10, and β-actin Abs (Santa Cruz Biotechnology, Paso Robles, CA; HRP-conjugated goat anti-rabbit and goat anti-mouse IgG Abs (Jackson ImmunoResearch Laboratories, West Grove, PA). 2′,7′-Dichlorofluorescein diacetate (CM-H2DCFDA) was obtained from Invitrogen (Carlsbad, CA). Methyl-β-cyclodextrin (MCBD), N-acetyl-t-cysteine (NAC), and 5-azacytidine (5-Aza) were purchased from Sigma-Aldrich (St. Louis, MO). The concentrations of all the pharmacological inhibitors listed did not show any significant cytotoxic effects by themselves as confirmed by FACS analysis in each experiment. All other reagents were of the highest purity commercially available and were used as received.

Cells

Human intestinal epithelial (INT-407) and RAW 264.7 murine macrophage cells were purchased from American Type Culture Collection (Manassas, VA). INT-407 cells were grown at 37°C in 5% CO2 in α-MEM supplemented with 10% FBS and antibiotics (10 μM penicillin G and 10 μg/ml streptomycin). INT-407 cells have previously been used to evaluate the inflammatory process (22), cytotoxic effect (23), and cell adherence ability (24). Alternatively, RAW 264.7 cells were detached with 0.05% trypsin/EDTA and 1×105 cells were suspended in DMEM high glucose without L-glutamine (Sigma, St. Louis, MO). The level of endotoxin in 50 pg/ml rVvpE was <0.004 endotoxin units. Thus, we suggest that rVvpE is a purified recombinant protein containing a very low level of endotoxin and suitable for our experiments in this study.

MTT cell viability assay

Cell viability was determined using the conversion of MTT to formazan via mitochondrial oxidation. Cells were pretreated with indicated inhibitors prior to rVvpE exposure for various times. MTT solution was then added to each well at a final concentration of 1 mg/ml per well and the plates were incubated at 37°C for another 2 h. After incubation, 150 μl DMSO was added to each well to dissolve the formazan formed and the absorbance was read at 570 nm using a spectrophotometer.

5′-H-thymidine incorporation

The 5′-H-thymidine incorporation experiments were performed as previously described by Breit et al. (26). Briefly, cells were synchronized by serum starvation for 24 h and then exposed to 50 pg/ml rVvpE for 24 h. After the incubation period, 1 μCi [methyl-5-3H]thymidine (sp. act., 74 GBq/mmol, 2.0 Ci/ml; Amersham Biosciences, Buckinghamshire, U.K.) was added to the cultures for 1 h at 37°C. Cellular [3H]thymidine uptake was quantitated by liquid scintillation counting of harvested cellular material (Wallac, Turku, Finland). All values were converted from absolute counts to percentages of control.

Flow cytometry

INT-407 cells were synchronized in the G0/G1 phase by culture in serum-free media for 24 h before incubation of rVvpE. The necrotic cell death was detected with an Annexin V and propidium iodide (PI) staining kit (BD Biosciences) according to the manufacturer’s instructions. Briefly, the cells were detached with 0.05% trypsin/EDTA and 1×105 cells were resuspended with annexin V binding buffer. The cells were then stained with annexin V (25 μg/ml) and PI (125 ng/ml) and incubated for 15 min at room temperature in the dark. The sample was read by flow cytometry and analyzed using CXP software (Beckman Coulter, Brea, CA). Samples were gated to exclude debris (forward light scatter [FSC] area versus side scatter area), and then any cell doublets were excluded using FSC area versus FSC width analysis.

Apoptosis/necrosis detection

Apoptosis/necrosis detection was also detected using an apoptosis/necrosis detection kit (Abcam, Cambridge, MA) according to the manufacturer’s instructions. Briefly, the cells were treated with Apopxin green indicator as a phosphatidylserine marker and 7-aminoactinomycin D and were then incubated for 60 min at room temperature. After the cells were rinsed with ice-cold PBS, the level of cell death was examined using a luminometer (Victor3; PerkinElmer, Waltham, MA) and quantified by measuring absorbance at excitation and emission wavelengths of 490 and 525 nm for detection of Apopxin green indicator or at excitation and emission wavelength of 490 and 650 nm for detection of 7-aminoactinomycin D.

Small interfering RNA transfection

Cells were grown until 75% of the surface of the plate and transfected for 36 h with ON-TARGETplus small interfering RNAs (siRNAs) mixed by four different siRNAs specific for ANXA2, ANXA4, or NF-kBp65 (GE Dharmacon, Lafayette, CO) or nontargeting siRNA as a negative control (GE Dharmacon) with HiPerFect transfection reagent (Qiagen, Valencia, CA). Four different siRNAs specific for ANXA2, ANXA4, or NF-kBp65 (GE Dharmacon, Lafayette, CO) were used to transfect the cells. To find the functional role of VvpE in INT-407 cells, we have previously grown INT-407 cells at 37°C in 5% CO2 in serum starvation for 24 h and then exposed to 50 pg/ml rVvpE for 24 h. After the incubation period, 1 μCi [methyl-5-3H]thymidine (sp. act., 74 GBq/mmol, 2.0 Ci/ml; Amersham Biosciences, Buckinghamshire, U.K.) was added to the cultures for 1 h at 37°C. Cellular [3H]thymidine uptake was quantitated by liquid scintillation counting of harvested cellular material (Wallac, Turku, Finland). All values were converted from absolute counts to percentages of control.

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CA) according to the manufacturers’ instructions. The siRNA efficacy for ANXA2, ANXA4, caspase-1, NOD-, LRR-, and pyrin domain–containing 3 (NLRP3), and NF-κBp65 determined by Western blotting was 59, 62, 70, and 67%, respectively.

**RNA isolation and RT-PCR**

Total RNA was extracted using the RNasy Plus mini kit (Qiagen). Reverse transcription was carried out with 3 µg RNA using a Maxima RT premix kit (iNtRON Biotechnology, Sungnam, Korea). β-Actin was used as an endogenous control. The cDNA (5 µl) for the annexins family was amplified.

**Quantitative real-time PCR**

The real-time quantifications of proinflammatory cytokines were performed using a Rotor-Gene 6000 real-time thermal cycling system (Corbett Research, Mortlake, NSW, Australia) with a QuantiMix SYBR Kit (PhileKorea Technology, Daejeon, Korea) according to the manufacturers’ instructions. β-Actin was used as an endogenous control.

**IL-1β ELISA**

INT-407 and RAW 264.7 cells plated on 60-mm culture dishes were grown in FBS-free medium for 24 h and divided into groups according to the experimental protocol. The IL-1β concentration in the culture medium was quantified by an ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Immunoprecipitation and Western blot analysis**

Interaction of ANXA2 with NOX2, NCF1, or caveolin-1 was analyzed by immunoprecipitation and Western blotting. Cells were lysed with lysis buffer (1% Triton X-100 in 50 mM Tris-HCl [pH 7.4]) containing 150 mM NaCl, 5 mM EDTA, 2 mM Na3VO4, 2.5 mM NaF, 100 mM Na3PO4, 200 mM microcystin lysine–arginine, and protease inhibitors). Cells lysates (400 µg) were mixed with 10 µg of each Ab. The samples were incubated for 4 h, mixed with protein A/G Plus–agarose immunoprecipitation reagent (Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated for an additional 12 h. The beads were washed four times and the bound proteins were released from the beads by boiling in SDS-PAGE sample buffer for 5 min. Samples were analyzed by Western blotting, which was performed as described with minor modifications (27).

**Detergent-free purification of caveolin-rich membrane fraction**

INT-407 cells were washed twice with ice-cold PBS, scraped into 2 ml 500 mM sodium carbonate (pH 11.0), transferred to a plastic tube, and homogenized with a Sonicator 250 apparatus (Branson Ultrasonic, Danbury, CT) using three 20-s bursts. The homogenate was adjusted to 45% sucrose by the addition of 2 ml 90% sucrose prepared in MES-buffed solution consisting of 25 mM MES buffer solution (pH 6.5) and 0.15 M NaCl and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient was formed (4 ml each of 5 and 35% sucrose, both in MES buffer solution containing 250 mM sodium carbonate) and centrifuged at 40,000 × g for 20 h in a Beckman SW-41 Rotor (Beckman Coulter, Fullerton, CA). Twelve fractions were collected and analyzed by 12% SDS-PAGE.

**Intracellular reactive oxygen species detection**

CM-H2DCFDA was used to detect the general reactive oxygen species (ROS) production. To quantify the intracellular ROS levels, the cells treated with 10 mM DCF-DA were rinsed twice with ice-cold PBS and then scraped. A 100-µl cell suspension was loaded into a 96-well plate and examined using a luminometer (Victor3, PerkinElmer) and a fluorescent plate reader at excitation and emission wavelengths of 485 and 535 nm, respectively.

**Immunofluorescence analysis**

Either INT-407 cells or ileum frozen sections were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized in 0.1% Triton X-100 in PBS for 5 min, and blocked in PBS containing 5% (v/v) normal goat serum for 2 h. After washing with PBS, samples were mounted on slides and visualized with an Olympus Fluoview 300 confocal microscope with a ×400 objective.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChiP) was performed using the EZ-ChIP kit (EMD Millipore, Billerica, MA) according to the manufacturer’s protocol. Briefly, cells were treated with 1% formaldehyde for 15 min to cross-link proteins to DNA, lysed, and then sonicated. The lysate was incubated with primary Ab overnight at 4°C. The immunocomplex was purified by incubation with 60 µl protein G–agarose beads for 1 h and eluted for DNA purification. Quantitative real-time PCR (qRT-PCR) was performed with primers for the IL-1β promoter flanking the putative NF-κBp65 binding sites. The following primer sequences of IL-1β promoter were used (sense and antisense, respectively): –488 to –327 bp, 5'-GGTAGGACCCCAACCCTCA-3' and 5'-TGGGACAAAGTGGAAGAACAC-3' and 5'-TTGGAAGGCGAAGTAGT-3'. Anti-rRNA polymerase II and normal mouse IgG were used as the positive and negative control for immunoprecipitation, respectively. The human IL-1β promoter sequence was found using the Eukaryotic Promoter Database. The putative binding sites were predicted using Aliggen Promoter software, version 3.0.2 (28, 29).

**Methylation analysis**

Genomic DNA from INT-407 cells was prepared with the QIAamp DNA mini kit (Qiagen). The extracted DNA was treated with sodium bisulfite using an EzSeq DNA methylation detection kit according to the manufacturer’s instructions (Koma Biotech, Seoul, Korea). The methylation status of IL-1β gene in INT-407 cells was determined by methyl-specific PCR (MSP) analysis. We conducted MSP of IL-1β gene promoter at −299 CpG sites. The following primer sequences for the −299 CpG sites of IL-1β promoter were used (sense and antisense, respectively): methylated, 5'-TATAACATACTCAATTATTTATAGC-3' and unmethylated, 5'-TATATATTTAATTTTTTTTTTATAGC-3' and 5'-ACAAAAATACATTATTATTCCATCGC-3' and 5'-TACAAGTTATATTAATTTTATTTTATAGC-3'.

**Bacterial strains, plasmids, and culture media**

The strains and plasmids used in this study are listed in Table I. All *V. vulnificus* strains (*M06-24/O* and *M06-24/O vvpE*) are isogenic and naturally resistant to polymyxin B. Unless otherwise noted, *V. vulnificus* strains were grown in LB medium supplemented with 2.0% (w/v) NaCl and 0.5% (w/v) tryptone at 30°C. All media components were purchased from Difco Laboratories (Detroit, MI). *V. vulnificus* was grown to midlog phase (A600 of 0.600) corresponding to 2 × 10⁹ CFU/ml and centrifuged at 6000 × g for 5 min. The pellet was washed with PBS and adjusted to desired CFU/ml based on the A600 determined using an UV–visible spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) to estimate culture density.

**Mouse model and colonization assay**

All animal procedures were performed following the National Institutes of Health guidelines for the humane treatment of animals, with approval from the Institutional Animal Care and Use Committee of Seoul National University (SNU-1401084). Seven-week-old mice (n = 10) were received intragastrically (g) inoculation of boiled *V. vulnificus* (control), *V. vulnificus* (wild-type [WT]), and a mutant deficient in vvpE gene in *V. vulnificus* (*M06-24/O vvpE*) at 1.1 × 10⁹ CFU/ml for 18 h and sacrificed. Mice ileum tissue was collected, washed, and homogenized. The homogenates of each organ were serially diluted and spread on LB agar containing polymixin B (100 µl/ml). CFUs were normalized to grams of intestinal tissues (CFU/g) to represent superficial bacterial counts. Note that the mice were given Bdu (50 mg/kg) by i.p. injections for 2 or 48 h prior to the sacrifice. Half of ileum tissues were naturally rinsed with ice-cold PBS and stored at −70°C for protein and RNA analysis. The other half were embedded in OCT compound and stored at −70°C. Samples were then cut into 6-µm-thick sections.

**Wound-healing migration assay**

INT-407 cells were seeded at 4 × 10⁴ cells on low 35-mm dishes with both silicone reservoirs, which are separated by a 500-µm-thick wall (Ibidi, Martinsried, Germany) (30) and incubated until the cell reached ∼100% confluence in serum containing medium. After serum starvation, the silicone reservoirs were removed with sterile forceps to create a wound field. The cells were incubated for an additional 6 h with vvpE (50 µg/ml) in the presence of 1% FBS and visualized with an Olympus Fluoview 300 confocal microscope with a ×100 objective.

**Oris cell migration assay**

INT-407 cells were seeded at 3 × 10⁴ cells/100 µl in an Oris well (Platypus Technologies, Madison, WI) and incubated for 24 h to permit cell
adhesion. When the cell reached ~70% confluence, cells were then incubated with serum-free medium and the inserts were carefully removed. The cells were incubated for an additional 6 h with rVvpE (50 pg/ml) in the presence of 1% FBS and visualized with an Olympus FluoView 300 confocal microscope. After 1 h incubation, more than 80% of the detached cells and the cell debris were quantified by using a microplate reader at excitation and emission wavelengths of 485 and 515 nm, respectively.

Host cell infection protocol

INT-407 and RAW 264.7 cells (1 × 10^6 cells/ml) grown to confluence in 6-well plates were incubated in serum/antibiotic-free media for 24 h. The bacterial suspensions in serum/antibiotic-free media were added to the cells at a multiplicity of infection (MOI; ratio of number of bacteria to number of epithelial cells) of 1, 5, and 10, after which the infected cells were incubated for 1 h. For IL-1β ELISA, co-culture supernatants were obtained and harvested by centrifugation at 4000 × g for 10 min at 4˚C. This was repeated once more with the resulting supernatant. The clarified supernatants were filtered through a 0.22-μm pore membrane to ensure the removal of any remaining bacterial cells and debris. For IL-1β qRT-PCR, total RNA was extracted from the INT-407 and RAW 264.7 cells.

Statistical analysis

Results are expressed as mean ± SEM. All experiments were analyzed by ANOVA, followed in some cases by a comparison of treatment means with a control using the Bonferroni–Dunn test. Differences were considered statistically significant at p < 0.05.

Results

VvpE induces proinflammatory response during the necrosis of intestinal epithelial cells

To find the functional role of VvpE, human intestinal epithelial (INT-407) cells were exposed to various concentrations (0–200 pg/ml) of rVvpE for 6 h. rVvpE significantly induced cytotoxicity of INT-407 cells from 50 to 200 pg/ml, compared with the cells with no treatment (Fig. 1A). An increase in cytotoxicity was observed after 6 h of incubation with 50 pg/ml rVvpE (Fig. 1B). The results after the [3H]thymidine incorporation of INT-407 cells also revealed that 50 pg/ml rVvpE significantly attenuated the level of DNA synthesis, compared with the vehicle (Fig. 1C). We further performed flow cytometric analyses using PI and annexin V for identification of necrotic and apoptotic cells caused by rVvpE treatment (Fig. 1D). rVvpE significantly induced the necrotic cell death (a 5.1 ± 0.3-fold increase compared with the vehicle) of INT-407 cells, whereas for apoptotic cell death, this shows a marginal effect (a 1.9 ± 0.3-fold increase compared with the vehicle). We further confirmed the necrosis-promoting effect of rVvpE by using another reagent that monitors the apoptotic cells with annexin V as well as the necrotic cells with 7-aminoactinomycin D, a fluorescent membrane-impermeable red dye, which has strong affinity GC-rich regions of DNA. As shown in Fig. 1E, we found that rVvpE has relatively more stimulatory potency on the necrotic cell death than on the apoptotic cell death, confirming that rVvpE is essential for triggering the necrotic cell death rather than the apoptosis. This result suggests that the functional role of rVvpE to induce cell death is reproducible in different assays. We further assessed whether rVvpE evokes proinflammatory response, which is typically caused by necrotic cell death. rVvpE increased the expression of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α from 6 h in INT-407 cells (Fig. 1F). Among the proinflammatory cytokines, the induction of IL-1β was particularly susceptible to the rVvpE treatment and its mRNA expression was significantly upregulated from 2 h after incubation with 50 pg/ml rVvpE (Fig. 1G). We also explored the ability of rVvpE to regulate IL-1β production in an ELISA assay. In contrast to the control, 50 pg/ml rVvpE evoked a substantial induction of IL-1β secretion from 6 h (Fig. 1H). These data provide the important evidence that rVvpE regulates the expression and secretion of IL-1β during necrotic cell death in intestinal epithelial cells. To ensure the role of VvpE in IL-1β activation, INT-407 cells were exposed to various MOI (ratio of number of bacteria to number of host cells) of V. vulnificus (WT) and a mutant deficient in vvpE gene in V. vulnificus (VvpE mutant). An increase in the expression and production of IL-1β was observed at 1 h after incubation with 10 MOI of WT, whereas VvpE mutant negated the IL-1β activation (Fig. 1I). More than 80% of the detached cells and the cell debris were exhibited from 2 h after incubation with 10 MOI of WT (data not shown). The VvpE mutant at 10 MOI also has the ability to prevent the activation of IL-1β induced by WT in RAW 264.7 murine macrophage cells (Fig. 1J). This result suggests that VvpE plays a critical role in IL-1β activation and the functional role of VvpE to induce IL-1β activation is reproducible with other types of immune cells.

ANXA2 is required for the necrosis and inflammation induced by VvpE

Annexin As (ANXAs) are thought to confer diverse cellular processes such as cell growth and inflammation as multifunctional proteins. The expression of ANXAs on the cell surface is thought to serve as a receptor that regulates several membrane-related events, such as exosome/endocytosis (32) and membrane–cytoskeletal interaction (33). We first determined the existence of ANXA isotypes in INT-407 cells. ANXAs are expressed in the order of ANXA1, ANXA2, ANXA4, ANXA5, ANXA6 > ANXA8, ANXA9, ANXA10, ANXA11 > ANXA7 (Fig. 2A). The expression of ANXA3 and...
ANX13 were not detected in these cell lines. Because it was previously shown that ANXA2 and ANXA4 of all the ANXA isoforms are essential in pathogenesis of bacterial infection (34, 35), we then determined the involvement of ANXA2 and ANXA4 in the necrotic cells death elicited by rVvpE (Fig. 2B). Interestingly, the cytotoxic effect of rVvpE was significantly silenced by the knockdown of ANXA2. Knockdown of ANXA4 by siRNA did not show any significant effect on necrotic cell death in rVvpE-treated INT-407 cells. Additionally, IL-1β production by rVvpE was markedly inhibited by knockdown of ANXA2, not by

**FIGURE 1.** VvpE induces proinflammatory response during the necrosis of intestinal epithelial cells. (A) Dose responses of rVvpE for 6 h in MTT assay are shown. (B) Time responses of 50 pg/ml rVvpE in MTT assay are shown. (C) [3H]thymidine incorporation was determined by treatment with 50 pg/ml rVvpE for 6 h in INT-407 cells. (D) Cells were incubated with rVvpE for 6 h. Percentages of necrosis, survival, and apoptosis were measured by using PI/annexin V staining and flow cytometry (left panel). PI/annexin V cells (Q1) were considered necrotic, PI/annexin V double-positive cells (Q2) were considered late apoptotic, PI/annexin V cells (Q3) were considered alive, and PI/annexin V cells (Q4) were considered early apoptotic. Quantitative analysis of the fold changes of necrotic (Q1) and apoptotic (Q2+Q4) cells by FACS analysis is shown (right panel). (E) Quantitative analysis of the fold changes of apoptotic and necrotic cells by an apoptosis/necrosis detection kit is shown. (F) Expression of proinflammatory cytokines is shown. Time responses of rVvpE in IL-1β expression and IL-1β secretion were evaluated by qRT-PCR (G) and ELISA (H), respectively. IL-1β expression and IL-1β secretion in INT-407 (I) and RAW 264.7 cells (J) infected with wt and VvpE mutant at 1, 5, and 10 MOI for 1 h are shown. (A–H) Data represent mean ± SEM. n = 5. *p < 0.05 versus control or vehicle (Veh, boiled rVvpE, 50 pg/ml). (I and J) Data represent mean ± SEM n = 5. *p < 0.05 versus control. #p < 0.01 versus 10 MOI of WT.
ANXA4 (Fig. 2C). These results suggest that ANXA2 is critically involved in proinflammatory response during the necrosis. Cholesterol in plasma membrane has been thought to be one of the cellular receptors of *V. vulnificus* (36). To confirm the structural importance of membrane rafts in the rVvpE-mediated signaling pathway, we employed the lipid raft sequester MβCD, which is known to deplete cholesterol from the cell membrane. Interestingly, MβCD significantly attenuated the necrotic cell death (a 2.7 ± 0.5-fold decrease compared with the rVvpE alone) (Fig. 2D) as well as the production of IL-1β in INT-407 cells (Fig. 2E), suggesting that rVvpE in acting through lipid raft is essential for triggering the proinflammatory signaling pathway during the necrotic cell death. We then determined how rVvpE links to the activation of ANXA2 within lipid raft by means of discontinuous sucrose density-gradient centrifugation. Fig. 2F shows that the lipid raft markers caveolin-1 and flotillin-2 were found in mainly fraction 6. ANXA2 was highly enriched in fractions 9–12 representing non–lipid raft parts of membrane. Interestingly, an increase in the level of ANXA2 appeared in fractions 5 and 6 after incubation with 50 pg/ml rVvpE compared with the vehicle alone, suggesting that rVvpE enhances the recruitment of ANXA2 into lipid rafts. Moreover, the subunits of NOX enzymes, NOX2 (gp91phox) and NCF1 (p47phox), were highly enriched in fractions 10–12. However, rVvpE treatment resulted in translocations of NOX2 and NCF1 into fraction 6, including lipid rafts part. The effect of rVvpE on the membrane location of ANXA2 was further visualized by staining the ANXA2 and lipid raft marker molecule, cholera toxin subunit B (CTB). As shown in Fig. 2G, rVvpE significantly increased the colocalization of CTB with ANXA2. Because the above approaches are qualitative at best, we further tried to quantify the results by using coimmunoprecipitation of ANXA2 with proteins related to the lipid rafts in the presence of...
rVvpE. It was noted that ANXA2 coimmunoprecipitated with caveolin-1, NOX2, and NCF1, and, importantly, that these interactions were enhanced by the rVvpE treatment (Fig. 2H). This means that lipid raft–mediated clustering of ANXA2, NOX2, and NCF1 is required to trigger the bacterial signaling pathway induced by rVvpE in INT-407 cells.

Regulatory effect of VvpE on ROS production and NF-κB phosphorylation

Clustering of NOX enzymes facilitates the production of ROS, which results in a prominent amplification of the transmembrane signal (37). A significant increase in the ROS level appeared after incubation with 10–50 pg/ml for 30 min compared with the vehicle alone (Fig. 3A, left panel). The increase in ROS production was transiently augmented between 0.5 and 1 h after incubation with 50 pg/ml rVvpE (Fig. 3A, right panel). Additionally, a pretreatment with MβCD as well as silencing of ANXA2 significantly blocked rVvpE-induced ROS production (Fig. 3B). Antioxidant NAC was used as a positive control. The inhibitory effects of MβCD and ANXA2 siRNA on ROS production were further visualized by staining INT-407 cells with a fluorescent dye, CM-H2DCFDA (Fig. 3C). Consistently, pretreatment with the NAC significantly blocked the necrotic cell death (Fig. 3D) as well as the production of IL-1β in INT-407 cells (Fig. 3E). These data suggest an involvement of ROS production in promoting necrotic cell death and proinflammatory response induced by rVvpE. We further examined the role of rVvpE in activation of NF-κB, which is a direct transcriptional target for necrotic signaling pathway as well as inflammation. As shown in Fig. 3F, NF-κBp65 phosphorylation increased between 1 and 3 h after incubation with 50 pg/ml rVvpE. The increased accumulation of NF-κBp65 phosphorylation in the nucleus was further confirmed by immunofluorescence staining and counterlabeling with PI (Fig. 3F, right panel). Additionally, the phosphorylation of NF-κBp65 evoked by a treatment with rVvpE was markedly inhibited by pretreatment with the MβCD and

FIGURE 3. Regulatory effect of VvpE on ROS production and NF-κB phosphorylation. (A) Dose responses of rVvpE for 1 h (left panel) and time responses of 50 pg/ml rVvpE (right panel) in ROS production are shown. n = 4. Cells were transfected with ANXA2 siRNA for 24 h or pretreated with MβCD (0.1 mM) and NAC (10 μM) prior to rVvpE exposure for 1 h. (B) The level of ROS production is shown. n = 4. (C) ROS production (green) was visualized by confocal microscopy. Scale bars, 100 μm. n = 3. Cells were pretreated with NAC prior to rVvpE exposure for 6 h. (D) FACS analysis and quantitative analysis of the percentage of necrotic (Q1) cells are shown. n = 4. (E) The level of IL-1β protein was quantified by ELISA. n = 5. (F) Time responses of rVvpE in the phosphorylation of NF-κBp65 are shown (left panel). p–NF-κBp65 (green) was detected by immunostaining with p–NF-κBp65 Ab in cells treated with rVvpE for 2 h (right panel). Scale bars, 50 μm (original magnification ×400). PI was used for nuclear counterstaining (red). n = 3. (G) Cells were pretreated with MβCD or NAC prior to rVvpE exposure for 2 h. The phosphorylation of NF-κBp65 is shown. n = 4. (H) Cells were transfected with ANXA2 siRNA prior to rVvpE exposure for 2 h. The phosphorylation of NF-κBp65 is shown. n = 4. INT-407 cells transfected with NF-κBp65 siRNA were incubated with rVvpE (50 pg/ml) for 6 h. (I) FACS analysis and quantitative analysis of the percentage of necrotic (Q1) cells are shown. n = 4. (J) The level of IL-1β protein was quantified by ELISA. n = 5. (A, B, and D–J) Data represent mean ± SEM. *p < 0.01 versus control, vehicle alone, or nontargeting siRNA plus vehicle. †p < 0.05 versus rVvpE. Cont, control; Veh, vehicle.
NAC (Fig. 3G) as well as knockdown of ANXA2 (Fig. 3H). These data represent evidence that the phosphorylation of NF-κBp65 is regulated by ROS produced by the clustering of NOX enzymes and ANXA2 in membrane lipid rafts. Knockdown of NF-κBp65 by siRNA also showed a significant inhibitory effect on the necrotic cell death (Fig. 3I) as well as the production of IL-1β (Fig. 3J) in INT-407 cells.

**Regulatory effect of VvpE on the interaction of NF-κB with the IL-1β promoter**

To determine whether NF-κB directly regulates IL-1β mRNA expression, we performed IL-1β promoter region analysis using Algen Promoto (28, 29). The IL-1β promoter located between the transcription start site and −500 bp upstream contains two putative NF-κB binding sites and three methylation sites, including one site that was placed next to methylation sites (Fig. 4A). We first performed a ChIP assay followed by qRT-PCR in INT-407 cells treated with rVvpE to determine the regulatory effect of VvpE on the binding of NF-κB to the IL-1β promoter. We tested two sets of primers that include two putative NF-κB binding sites at the region proximal to −500 bp of the start site of the IL-1β promoter. We found that all primer sets result in an amplicon from the anti-pNF-κB65 immunoprecipitates, and, importantly, that the interaction of NF-κB with the IL-1β promoter was enhanced by the rVvpE treatment in only the primer set ranging between −347 and −174 bp (Fig. 4B). Interestingly, however, the level of NF-κB binding to the IL-1β promoter was significantly inhibited by knockdown of ANXA2 and by pretreatment with the MβCD and NAC (Fig. 4C, 4D). These results suggest that rVvpE transcriptionally regulates the NF-κB binding to the unique region of the IL-1β promoter, which was regulated by necrotic signaling pathway as well as inflammation. Because of the inhibitory role of promoter methylation on the gene transcription, we postulated that rVvpE could affect the methylations, which negatively regulate the interaction of NF-κB with the IL-1β promoter. We determined the methylation status following the treatment with rVvpE by means of MSP. A bisulfite treatment converted cytosine residues in the genomic DNA to uracil, which were amplified as thymine during the subsequent PCR. We attempted to analyze the methylation status of the IL-1β gene promoter at −299 Cpg sites due to their close distance to putative NF-κB binding sites. As shown in Fig. 4E, primers for −299 Cpg sites that specifically amplified either the methylated or unmethylated form of the IL-1β promoter produced 100-bp methylated bands from vehicle-treated INT-407 cells, indicating that the IL-1β promoter was methylated in these alleles. However, treatment with 50 pg/ml rVvpE for 2 h markedly inhibited the level of IL-1β promoter methylation, which was significantly blocked by knockdown of ANXA2 and by pretreatment with the MβCD and NAC. Interestingly, the levels of the phosphorylation of NF-κBp65 (Fig. 4F) and production of IL-1β (Fig. 4G) were markedly enhanced by pretreatment with the DNA methylation inhibitor 5-Aza. These data indicate that the hypomethylation of the IL-1β promoter is able to affect the high level of the interaction of NF-κB with the IL-1β promoter during necrotic cell death evoked by rVvpE. *V. vulnificus* was known to stimulate the activation of caspase-1 and IL-1β via the NLRP3 inflammasome, which is one member of NLR (38). Similar to the previous report (38), an increase in active forms of IL-1β (17 kDa) and caspase-1 (10 kDa) was observed after 6 h of incubation with 50 pg/ml rVvpE (Fig. 4H). However, the activation of IL-1β by rVvpE was markedly inhibited by transfection with siRNAs for NLRP3 and caspase-1 (Fig. 4I). Thus, these results indicate that rVvpE has the ability to induce pyroptosis accompanied by NLRP3-mediated caspase-1 activation that results in maturation of IL-1β.

**Essential role of VvpE in promoting inflammation-related intestinal injury**

To evaluate the functional relevance of *V. vulnificus* VvpE, 7-wk-old ICR mice were inoculated i.g. with boiled *V. vulnificus* (control), *V. vulnificus* (WT), and a mutant deficient in vvpE gene in *V. vulnificus* (vvpE mutant) at 1.1 × 10⁶ CFU/ml for 18 h, after which the level of bacterial colonization was monitored. The WT colonization in ileum increased by 3.2 ± 0.3 (×10⁶ CFU/g tissue) (Fig. 5A). However, when the mice were inoculated with vvpE mutant, the levels were diminished by 2.4 ± 0.2 (×10⁶ CFU/g tissue), compared with the WT. We found that all of the mice given oral administrations of WT had survived by 18 h postinfection (data not shown). These results indicate that VvpE contributes to the intestinal colonization of *V. vulnificus* in vivo. We further found that WT strongly induces the phosphorylation of NF-κBp65 (Fig. 5B) and the expression of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α (Fig. 5C) in the mouse intestine. However, unlike WT, the mice infected with vvpE mutant almost completely maintained the levels of NF-κBp65 phosphorylation and proinflammatory cytokines to the normal level. These results indicate that VvpE is a critical elastase that promotes intestinal inflammatory responses. In response to an inflammation-related injury, intestinal epithelial cells at the wound edge proliferate and migrate to cover denuded surfaces and re-establish the critical barrier function (39). Thus, we have further investigated whether VvpE also has a virulence effect on cell proliferation during inflammatory processes. We found that the number of proliferating cells identified by Ki67 labeling was significantly decreased in the intestinal crypt of mice infected with WT compared with control mice (Fig. 5D). However, the cytotoxic effect of WT was negated by infection with the vvpE mutant, suggesting that VvpE regulates host cell death as well as inflammatory response in intestinal epithelial cells. To assess the level of enterocyte migration, BrdU pulse chase experiments were performed in mice inoculated i.g. with control, WT, or vvpE mutant at 1.1 × 10⁶ CFU/ml for 18 h (Fig. 5E). Two hours after the BrdU injection, BrdU-labeled intestinal epithelial cells of control mice were located at the bottom of the ileum crypt. Subsequently, the cells migrated upward and moved farther along the crypt villus axis, reaching about two thirds of the location of the villi at 48 h. In comparison, mice infected with WT had fewer BrdU⁺ intestinal epithelial cells present at the ileum crypt and showed a reduced rate of migration compared with the control. Interestingly, the mice that received the vvpE mutant were still keeping the migratory behavior of intestinal epithelial cells at physiological levels. Thus, these results indicate that VvpE plays an important role in inflammation-related injury related to cell proliferation and migration in intestinal epithelial cells. To verify the role of VvpE in wound recovery, we further examined the ability of 50 pg/ml rVvpE to regulate cell motility for 6 h using an in vitro wound-healing migration assay. As shown in Fig. 5F, 1% FBS significantly induced cell motility of INT-407 cells. In contrast, the cell migration–promoting activity of FBS was attenuated by a rVvpE treatment. We also visualized the ability of VvpE to regulate cell motility by staining the cells with calcein AM in an Oris cell migration–promoting activity of FBS, which was inhibited by a rVvpE treatment. Additionally, Fig. 5H shows that rVvpE inhibited FBS-induced cell migration, but this effect was silenced by the knockdown of the expression of ANXA2, but not by ANXA4.
Discussion

Our data in the present study demonstrate that VvpE is the functional elastase of V. vulnificus that stimulates IL-1β secretion by means of hypomethylation and NF-κB activation via lipid raft–dependent ANXA2 recruitment and ROS signaling during the pyroptosis of intestinal epithelial cells. The mechanism and outcome of pyroptosis are distinctly different from these aspects of apoptosis, which actively inhibit inflammation. We showed that rVvpE induces pyroptotic cell death coupled with the production of IL-1β via lipid rafts. These findings differed from those previously reported and revealed that the effect of that VvhA, one of the major toxins of V. vulnificus, on cytotoxicity is independent of the action of lipid rafts (40). Thus, the results of our present study suggest that V. vulnificus uniquely regulates intestinal cell death and inflammation by producing the VvpE with modes of action that differ from those of other secreted proteins. In fact, many...
studies have reported that several enteric bacterial pathogens, including *H. pylori* vacuolating toxins (41) and the enterotoxin *Clostridium perfringens* (42), may interact with a detergent-resistant cellular membrane composed of relatively abundant cholesterol as an initial attachment platform and therefore having a cytotoxic effect on intestinal physiological functions. Therefore, the findings in the present study suggest that lipid rafts are a functional mediator that initiates the virulence effect of rVvpE to induce host cell death as well as inflammation. Concerning the pathogenic mechanism of VvpE, we subsequently showed the unique involvement of lipid raft–dependent ANXA2 recruitment and ROS production in the regulation of IL-1β secretion during pyroptotic cell death. The importance of ANXA2 in microbial pathogenesis is underscored by the finding that *E. coli* (*EPEC*) adherence induces the concentration of cholesterol and GPI-anchored proteins at sites of bacterial contact, where ANXA2 is recruited to the cytoplasmic membrane surface, possibly stabilizing raft patches and their links to the actin cytoskeleton beneath adhering to EPEC (34). Thus, these results present the possibility that rVvpE influences the structure and localization of ANXA2 through lipid rafts to promote proinflammatory signaling in intestinal epithelial cells. Compelling evidence further supported the critical role of ANXA2 in inflammatory myopathies (43) as well as in inflammatory bowel disease (44). Taken together, our finding that VvpE induces proinflammatory responses via an ANXA2-dependent mechanism provides information about the important mechanisms of pathogen-induced cell death during *V. vulnificus* infection. Increasing evidence has suggested that lipid rafts are clustered to form a redox signaling platform through gp91 phox (NOX2) coupling with cytosolic factors that include p47 phox (NCF1), p67 phox (NCF2), and small GTPase Rac1 (45-47), and that these processes subsequently produce superoxides and other ROS (45). Although in most cell types, mitochondrial ROS are thought to be the largest contributor to intracellular ROS production (48), our result showed that the sequestration of cholesterol by MβCD attenuates intracellular ROS production and
pyroptotic cell death induced by rVvpE. Hence, the current findings further indicate that epithelial ROS generated by NOX within lipid rafts may influence on the mitochondrial ROS production and thereby contribute to increased total intracellular ROS generation, leading to pyroptotic cell death.

The ROS generated by a bacterial infection have been shown to induce the oxidative inactivation of several proteins harboring oxidant-sensitive thiol groups and of the ubiquitin—proteasome pathway (16), thereby activating many redox-sensitive proteins that include regulators of MAPK pathways and focal adhesion kinase, as well as several components involved in NF-κB activation (16). NF-κB is one of the master oxidative transcriptional regulators of the host inflammatory signaling pathway and the cell death pathway in response to bacterial pathogens by inducing the expression of a proinflammatory mediator that orchestrates and sustains the inflammatory response and causes tissue damage (1, 16). We found that rVvpE can induce the phosphorylation of NF-κB through ROS production and that the inhibition of NF-κB blocks rVvpE-induced cell death. Regarding the role of ROS in NF-κB activation, earlier work showed that the JNK pathway induced by ROS can influence NF-κB activation in promoting apoptotic cell death (49). Additionally, pERK1/2 was reported to have the ability to translocate into the nucleus, where it phosphorylates various substrates, such as transcriptional factors, thereby transmitting the signals received by cell surface receptors to the nucleus (50). Hence, it is conceivable that ROS induced by rVvpE have a potential role in promoting the NF-κB pathway through the activation of ERK1/2 and JNK. Based on these results, we suggest that rVvpE stimulates ROS-mediated NF-κB activation in promoting the cell death of INT-407 cells. Whereas the NF-κB binding activity of the IL-1β gene promoter is the critical mechanism of the transcriptional regulation of IL-1β, the promoter methylation of cytosome residues at CpG dinucleotides is also an important posttranslational modification affecting NF-κB transcription. The important finding of this study is that rVvpE uniquely regulates the interaction between NF-κB and the IL-1β promoter at the region between −297 and −286 bp upstream of the transcription start while also inhibiting the methylation status of the IL-1β gene promoter at the −299 CpG site, located immediately before the −297 bp site occupied by NF-κB. It is not clear whether the regulatory effect of rVvpE in promoting the interaction between NF-κB and the IL-1β promoter is a sequential result of the hypomethylation of IL-1β promoter or, alternatively, an independent process involving other cellular signaling events. Although the effects of ROS on epigenetic modulation are still poorly understood, it was clearly shown that ROS induced by a H2O2 treatment regulate histone acetytransferases and deacetylases, the key enzymes responsible for chromatin remodeling, suggesting that posttranslational modifications affecting NF-κB are also redox sensitive (51). Indeed, our data revealed that hypomethylation by 5-Aza is closely related to high level of the NF-κB activation and to IL-1β secretion. These data provide, to our knowledge, the first evidence that a bacterial pathogen induces the hypomethylation of the IL-1β promoter as well as the transcriptional regulation of NF-κB to promote IL-1β production during host cell death.

Given current in vitro observations that IL-1β production is a hallmark of necrosis evoked by rVvpE, our in vivo study shows that VvpE is an important secreted virulence factor of V. vulnificus responsible for the inflammatory response, which stimulates NF-κB activation and proinflammatory cytokine expression. Although there are many limiting factors to understand why VvpE mutant completely inhibits the NF-κB activation and proinflammatory cytokine expression, it is possible that the VvpE would affect the delivery of major toxins (e.g., VvhA, MARTXVv) for NF-κB activation during intestinal infection of V. vulnificus. Alternatively, exacerbation of the inflammatory response in the intestine may reflect the altered proliferation and migration of intestinal epithelial cells during intestinal wound repair (39). Similarly, a previous study showed that an intestinal infection of H. pylori inhibits cell proliferation without affecting cell migration, thereby delaying wound healing in intestinal inflammatory injuries (52). However, the role of VvpE in promoting inflammation-related events is not yet well understood. The salient finding of this study is that VvpE is a functional elastase delaying the upward movement of enterocytes along the crypt/villus axis while also impairing enterocyte proliferation at the crypt part. These data further indicate that severe inflammation mediated by VvpE may interfere with epithelial cell migration and proliferation and that this may inhibit the reestablishment of the intestinal barrier function. In support of these findings, our in vitro results, verifying that rVvpE inhibits serum-induced cell migration via an ANXA2-dependent mechanism, provides good evidence that necrotic cell death may result in a delay of wound recovery by rVvpE. Indeed, an earlier study suggested that the silencing of ANXA2 enhances the proliferation of colon epithelial cells and ameliorates gut inflammation, improving intestinal mucosal wound repair (44). Hence, we suggest that ANXA2 plays a critical role as a host mediator of VvpE and that it has unique biological properties responsible for host cell death coupled with an inflammatory response.

In conclusion, our results suggest that rVvpE induces NF-κB-dependent IL-1β production via lipid raft-dependent ANXA2 recruitment and ROS production in intestinal epithelial cells. Thus, highlighting the signaling pathways involved in the rVvpE-stimulated cell death pathway may provide potential therapeutic targets for strategic modulations of V. vulnificus infections.

Disclosures

The authors declare no conflict of interest.

References


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