***Vibrio vulnificus* VvhA induces autophagy-related cell death through the lipid raft-dependent c-Src/NOX signaling pathway**

Eun Ju Song[1, +], Sei-Jung Lee[1, +], Hyeon Su Lim[1], Jun Sung Kim[1], Kyung Ku Jang[2], Sang Ho Choi[2], and Ho Jae Han[1,*]

Supplementary Materials and Methods

Purification of the recombinant haemolysin VvhA. To find the molecular role of VvhA in Caco-2 cells, we prepared a recombinant protein of VvhA (rVvhA) following the method of Lee et al.[1]. Briefly, the coding region of *vvhA* was amplified and then cloned into a His6-tag expression vector, pET29a(+) (Novagen, Madison, WI, USA), resulting in pKS1201 (Supplementary Table S1). *E. coli* BL21 (DE3) carrying the pKS1201 was grown in LB-ampicillin media at 37 °C until reaching an A_{600} between 0.5 and 0.6. For inducing the protein expression, the temperature was lowered to 30 °C and the culture treated with 1 mM isopropyl-β-D-thiogalactopyranoside for 6 h. The cells were harvested and prepared for isolating the soluble fraction. Cell lysate containing His_6-tagged VvhBA protein was mixed with 1 ml of Ni-NTA agarose (Qiagen, Valencia, CA) for 1 h at 4 °C and the mixture was loaded on Bio-Spin Chromatography Columns (Bio-Rad Laboratories, Hercules, CA, USA). The resin was washed with buffer A, and bound VvhBA protein was eluted with buffer A containing 300 mM imidazole. After purification, the homogeneity of VvhBA was assessed by 12 % SDS-PAGE and Coomassie Blue staining. Purified proteins containing rVvhA was dialyzed against 20 mM Tris-Cl, pH 8.0, concentrated to 0.3 mg/mL using Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific, Hudson, NH, USA), and stored at −80 °C until use.

Bacterial strains, plasmids, and culture media. The strains and plasmid used in the present study

are listed in Supplementary Table S1. All *V. vulnificus* strains (M06-24/O WT and M06-24/O *vvhA*) are isogenic and naturally resistant to polymyxin B. *V. vulnificus* strains were cultured in LB medium supplemented with 2.0 % (w/v) NaCl (LBS) at 30 °C. All media components were obtained from Difco Laboratories (Detroit, MI, USA). The bacterial strains were grown to mid-log phase (A_{600} = 0.500) corresponding 2×10^8 CFU/mL and centrifuged at 6000 × g for 5 min. The pellet was washed with PBS and prepared to desired colony-forming unit (CFU)/mL based on the A_{600} determined using a UV–VIS spectrophotometer (UV-1800, Shimadzu, Japan).

Complementation of the *vvhA* mutant. To complement the *vvhA* mutation, an open reading frame (ORF) of *vvhA* was amplified from the genomic DNA of *Vibrio vulnificus* MO6-24/O by PCR with the primer pair VVHA001F and VVHA001R (Supplementary Table S2) and then digested with BamHI. The amplified *vvhA* ORF was subcloned into the broad-host-range vector pRK415 linearized with the same enzyme (Supplementary Table S1) to result in pKK1449[2]. *Escherichia coli* S17-1 *λ pir, tra* strain containing pKK1449 was used as a conjugal donor to *vvhA* mutant[3]. The plasmid pKK1449 was delivered into the *vvhA* mutant by conjugation as described previously[4].

References for Supplementary

1. Lee, S. et al. *Vibrio vulnificus* VvhA induces NF-κB-dependent mitochondrial cell death via lipid raft-mediated ROS production in intestinal epithelial cells. Cell death & disease 6, 1655 (2015).

2. Keen, N., Tamaki, S., Kobayashi, D. & Trollinger, D. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene 70, 191-197 (1988).

3. Simon, R., Priefer, U. & Pühler, A. A broad host range mobilization system for invivo genetic-engineering-transposon mutagenesis in gram-negative bacteria. Bio-technology 1 (1983).

4. Park, J. H. et al. Correction: The cabABC Operon Essential for Biofilm and Rugose Colony Development in *Vibrio vulnificus*. PLoS pathogens 11, e1005252-e1005252 (2015).

Supplementary Table 1. Plasmids and bacterial strains used in this study

Strain or plasmid	Relevant characteristics [a]	Reference or source
Bacterial strains		
V. vulnificus		
M06-24/O	Clinical isolate; virulent; WT	Laboratory collection
E. coli		
BL21 (DE3)	F^- *ompT* $hsdS_B$ ($r_B^- m_B^-$) *gal dcm* (DE3)	Laboratory collection
S17-1λ*pir*	λ-*pir* lysogen; *thi pro hsdR* $hsdM^+$ *recA* RP4-2 Tc::Mu-Km::Tn7;Tp^r Sm^r; host for π-requiring plasmids; conjugal donor	Simon *et al*., 1983[3]
Plasmids		
pET29a(+)	His_6 tag fusion expression vector; Km^r	Novagen
pKS1201	pET29a(+) with VvhBA; Km^r	Lee *et al.*, 2015[1]
pRK415	IncP *ori*; broad-host-range vector, *oriT* of RP4; Tc^r	Keen *et al*., 1988[2]
pKK1449	pRK415 with *vvhA*; Tc^r	This study

[a] Tp^r, trimethoprim resistant; Sm^r, streptomycin resistant; Tc^r, tetracycline resistant

Supplementary Table 2. Sequences of primer pairs used in this study.

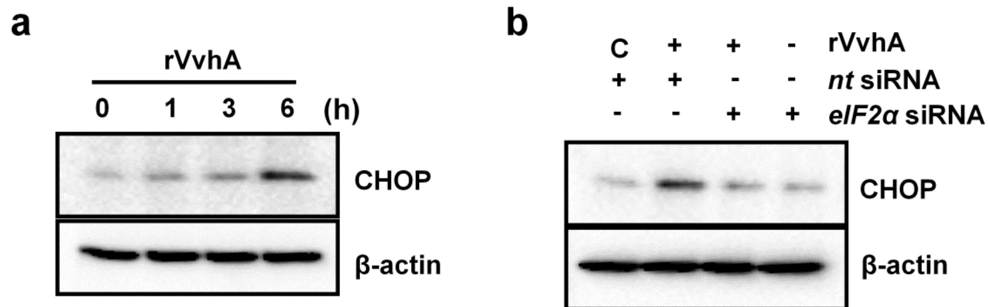
Genes	Forward primer/reverse primer
ULK1	5'-CAGAACTACCAGCGCATTGA-3' 5'-TCCACCCAGAGACATCTTCC-3'
ULK2	5'-CTCCTCAGGTTCTCCAGTGC-3' 5'-TTGGTGGGAGAAGTTCCAAG-3'
ATG14	5'-TCACCATCCAGGAACTCACA-3' 5'-TTCAGTCTTCGGCTGAGGTT-3'
VPS34	5'-ATGTGTATGGTCCCGGAAAA-3' 5'-TGTCGATGAGCTTTGGTGAG-3'
BECLIN-1	5'-GCCCTTTGAAGTACGAGCAG-3' 5'-GAGATCATCCCACCTGCACT-3'
ATG5	5'-CGGGAACACCAAGTTTCACT-3' 5'-TCTGGGGAGACATCCGTAAG-3'
ATG12	5'-TGGGATTGCAAAATGACAGA-3' 5'-TTCCCCATCTTCAGGATCAA-3'
ATG16L1	5'-GTCTTCGATGCACATGATGG-3' 5'-GATTCGGCTTGCAAAATCAT-3'
LC3B	5'-AGCAGCATCCAACCAAAATC-3' 5'-CTGTGTCCGTTCACCAACAG-3'
P62/SQSTM1	5'-CACCTGTCTGAGGGCTTCTC-3' 5'-CACACTCTCCCCAACGTTCT-3'
RAB7	5'-CTGACCAAGGAGGTGATGGT-3' 5'-CTGGCCTGGATGAGAAACTC-3'
FYCO1	5'-GGAGCTAGGAGCAGCAGAGA-3' 5'-CGCATCACTGGGAATAGGTT-3'
LAMP1	5'-CTTCAGCAGGGGAGAGACAC-3' 5'-TGTTGGGGTTGATGTTGAGA-3'
LAMP2	5'-GGTTAATGGCTCCGTTTTCA-3' 5'-ATGGGCACAAGGAAGTTGTC-3'
β-Actin	5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3' 5'-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3'

Supplementary Table 3. Oligonucleotides used in this study

Name	Oligonucleotide Sequence (5' → 3')[a, b]	Use
For mutant complementation		
VVHA001F	GGATCCAACCCTTACTCGTAATG	Amplification of the *vvhA* ORF
VVHA001R	GGATCCCTAGAGTTTGACTTGTTG	

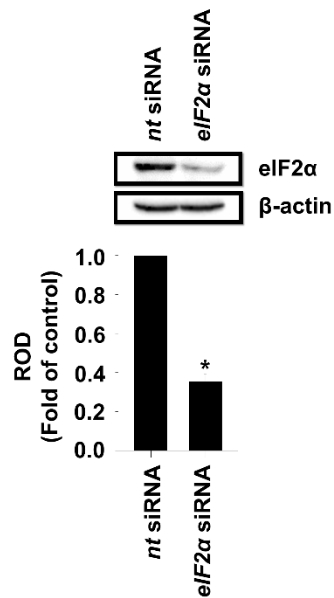
[a] The oligonucleotides were designed using the *V. vulnificus* MO6-24/O genomic sequence (GenBank™ accession number CP002469 and CP002470, www.ncbi.nlm.nih.gov).

[b] Regions of oligonucleotides not complementary to the corresponding genes are underlined.



Supplementary Figure 1. Effect of rVvhA on ER stress.

(a) Time responses of rVvhA in the expression of CHOP were confirmed by western blotting. n = 3. **(b)** Cells were transfected for 36 h with *eIF2α* or *non-targeting* (*nt*) siRNA using TurboFect Transfection Reagent prior to rVvhA exposure for 6 h. CHOP expression was analyzed by using Western blot. n = 3.



Supplementary Figure 2. Effect of siRNA on target protein. Cells were transfected for 36 h with *eIF2α* or *non-targeting* (*nt*) siRNA using TurboFect Transfection Reagent. Protein expressions were analyzed by using Western blot. The knockdown efficacies of eIF2α was 65%. Error bars represent the means ± S.E. *$P < 0.05$ vs *nt* siRNA