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Intestinal epithelial cell apoptosis due to a hemolytic toxin from *Vibrio vulnificus* and protection by a 36 kDa glycoprotein from *Rhus verniciflua* Stokes

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ABSTRACT

Rhus verniciflua stokes (RVS) has been used as a functional food to cure inflammatory diseases in Korea. In the present study, we carry out an investigation of the cellular mechanism of a 36 kDa glycoprotein isolated from RVS fruit (RVS glycoprotein) during the apoptosis of human gastrointestinal epithelial HCT116 cells induced by the hemolytic toxin (VvhA) produced by *V. vulnificus*. Recombinant protein (r) VvhA produced by *V. vulnificus* stimulated apoptosis by activating the phosphorylation of protein kinase C (PKC) through the production of intracellular reactive oxygen species (ROS). However, RVS glycoprotein significantly inhibited the level of ROS production and PKC activation in rVvhA-stimulated HCT116 cells. Interestingly, we found that RVS glycoprotein has inhibitory effects on the phosphorylation of c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB), which are responsible for the expression of Bax and cleaved caspase-3 in HCT116 cells treated with rVvhA, respectively. On the basis of these results, we suggest that RVS glycoprotein blocks mitochondrial apoptotic cell death induced by rVvhA via the inhibition of ROS-mediated signaling events in HCT116 cells.

1. Introduction

Vibrio vulnificus (*V. vulnificus*), an anaerobic Gram-negative marine bacterium, is an exceedingly virulent food pathogen that often causes gastrointestinal epithelial cell death, septicemia, and gastroenteritis (Jeong and Satchell, 2012). When host cells are infected with *V. vulnificus*, most virulence effects of *V. vulnificus* are mediated by virulent factors and enzymes, such as VvpM, MARTX, VvpE, and VvhA (Jeong and Satchell, 2012; Lee et al., 2007, 2014; Miyoshi, 2006). VvhA, a 51 kDa hemolytic pore-forming toxin, has been considered to be major exoprotease that causes cytotoxic effects and starting an apoptosis process in gastrointestinal epithelial cells (Wright and Morris, 1991). VvhA is actively produced *in vivo* during a *V. vulnificus* infection and is lethal to mice at the submicrogram level (Park et al., 1996). VvhA causes tachycardia and hypotension and has strong toxicological effects on the skin and pulmonary tissues in animals (Fan et al., 2001; Wright and Morris, 1991). VvhA is also known to induce apoptotic cell death via intracellular reactive oxygen species (ROS) production during the infection of host cells (Lee et al., 2015). Thus, it has been suggested that the toxicological intracellular ROS produced by host cells triggers cell death signaling cascades during *V. vulnificus* infections (Fang, 2011; Slauch, 2011).

Apoptosis is a process of programmed cell death that can be induced by many bacterial pathogens and is generally characterized by morphological characteristics such as reduced cell volumes, chemical-induced cell death, chromatin condensation, and nuclear DNA fragmentation (Hengartner, 2000). There are numerous apoptotic factors involved in a host infection of *V. vulnificus*, such as oxidative stress, the MAPKs signaling pathway, mitochondrial damage, and caspase-3 activation (Lee et al., 2015). Unfortunately, many antibiotic therapies against infections of *V. vulnificus* are not suitable for high-risk individuals with chronic liver diseases, immunodeficiency, or iron storage

Abbreviations: Bax, Bcl-2 associated X protein; CM-H_2DCFDA, 2′, 7′ -dichlorofluorescein diacetate; IPTG, isopropyl-β-dthiogalactopyranoside; JNK, c-Jun N-terminal kinase; MAPKs, mitogen-activated protein kinases; NAC, N-acetyl-L-cysteine; NF-κB, nuclear factor-kappa B; PKC, phosphorylation of protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; RVS, *Rhus verniciflua* stokes; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; *V. vulnificus*, *Vibrio vulnificus*

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illnesses (Horseman and Surani, 2011). In these contexts, many scientists have proposed that the increased dietary intake of antioxidants such as α-tocopherol, flavonoids, and phenolic compounds can reduce the risk of bacterial infections and have inhibitory effects on the production of reactive oxygen species (ROS), the activation of pro-apoptotic mediators, and excessive inflammatory pathological symptoms (Gonzalez-Ponce et al., 2018; Pandey and Rizvi, 2009). Therefore, the discovery and identification of safe new drugs, which are particularly applicable to high-risk individuals have become an important goal in research in the biomedical sciences.

Rhus verniciflua stokes (RVS) has traditionally been used as a food additive in herbal medicine, and for the healing of inflammatory diseases in Korea (Jeong et al., 1997; Lohakare et al., 2006). In earlier work, the authors demonstrated that ethanol crude extracts of RVS contain cellular active agents that can scavenge ROS and inhibit apoptotic cell death mediated by oxidative stress (Lee et al., 2002; Lim et al., 2003). In this context, we isolated a glycoprotein (36 kDa) consisting of carbohydrate (38.75%) and protein (61.25%) components from RVS fruit and reported that RVS glycoprotein has strong antioxidative activity and anti-apoptotic effects via the modulation of NF-κB and AP-1 activities in fibroblasts and breast epithelial cells (Ko et al., 2005). Having shown that RVS glycoprotein has many biological functions, we speculated that RVS glycoprotein may be effective against diseases involving considerable amounts of ROS production a potent causative factor in cancer, diabetes, and arteriosclerosis. In addition, there is no evidence of anti-apoptosis features by plant glycoproteins in the gut during *V. vulnificus* infections. Therefore, we examined the antioxidative and anti-apoptotic effects of RVS glycoprotein on HCT116 gastrointestinal epithelial cells treated with *V. vulnificus* VvhA.

2. Materials and methods

2.1. Chemicals

FBS was purchased from GE Healthcare (Logan, UT, USA). The following antibodies were purchased: PKC, phospho-PKC, ERK, phospho–ERK, c-Jun N-terminal kinase (JNK), phospho–JNK, p38, phospho–p38, NF-κBp65, phospho—NF—κBp65, Bax, Bcl-2, cleaved caspase-3, and β-actin antibodies (Santa Cruz Biotechnology, Paso Robles, CA); HRP-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies (Gene Tex, Irvine, CA, USA). 2′, 7′ -dichlorofluorescein diacetate (CM-H_2DCFDA) was obtained from Invitrogen (Carlsbad, CA, USA). N-acetyl-L-cysteine (NAC) was purchased from Tocris (KOMA Biotech, Seoul, Korea). Bisindolylmaleimide I, PD98059, SP600125 and Bay 11–7082 were purchased from MedChemExpress (Monmouth Junction, NJ, USA). The concentrations of all of the pharmacological inhibitors listed did not show any significant cytotoxic effects by themselves. All other reagents were of the highest purity commercially available and were used as received.

2.2. Cells

HCT116 gastrointestinal epithelial cells were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea) and cultured at 37 °C in 5% CO_2 in McCoy's medium containing 10% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin, respectively. The medium was renewed twice a week. These cell lines have previously been used to evaluate the function of virulence factors of *V. vulnificus* in the regulation of apoptotic process and cytotoxic effects (Lee et al., 2014, 2018).

2.3. Isolation of Rhus verniciflua Stokes (RVS) glycoprotein

Rhus verniciflua stokes (RVS) glycoprotein was isolated and purified from the fruits of *Rhus verniciflua* stokes, as described previously (Bermejo et al., 2005; Oh et al., 2006). Briefly, the fruits were broken into small pieces, and soaked in water for several months in a dark basement. The water extract was filtered through Whatman filter paper (No. 2) and concentrated with a rotary evaporator (Buchi, Flawil, Switzerland). The concentrated solution was dried with a freezer-dryer (Sam Won, Seoul, Korea). Five grams of dried-crude water extract was dissolved in distilled water. The solution was precipitated with 80% ammonium sulfate, dialyzed with a dialysis membrane (Spectra/por, MWCO 6000–8000, Pasadena, CA, USA) against 20 mM Tris–HCl (pH 7.4) overnight. After dialysis, the sample solution was dried with a freeze dryer and stored at -70 °C. The glycoprotein was verified using Schiff's reagent on the gel after electrophoresis and its purity was more than 95.0%. The dried sample 30.5 mg (0.61% from the original sample) was stored at -20 °C during the experimental period. After verification of high purity of glycoprotein, we treated into the cells for further studies.

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
CMM111	M06-24/O vvhA::Pks1201; elastase deficient; vvhA mutant	Jeong et al. (1997)
E. coli		
BL21 (DE3)	F⁻ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3)	Laboratory collection
Plasmids		
pET29a(+)	His_6 tag fusion expression vector; Km^r	Novagen
pKS1201	pET29a(+) with VvhBA; Km^r	This study

[a] Km^r, kanamycin resistant

2.4. Bacterial strains, plasmids, and culture media

The strains and plasmids used in this study are listed in Table 1. All *V. vulnificus* strains (M06-24/O and M06-24/O vvhA) are isogenic and naturally resistant to polymyxin B. Unless otherwise noted, *V. vulnificus* strains were grown in Luria Bertani medium supplemented with 2.0% (wt/vol) NaCl (LBS) at 30 °C. All media components were purchased from Difco (Difco Laboratories, Detroit, MI, USA). *V. vulnificus* were grown to mid-log phase (A_{600} = 0.500) corresponding to 2 × 10^8 CFU/ml and centrifuged at 6,000 × *g* for 5 min. The pellet was washed with PBS and adjusted to desired colony-forming unit (CFU)/ml based on the A_{600} determined using a UV–VIS spectrophotometer (UV-1800, Kyoto, Shimadzu, Japan) to estimate culture density.

2.5. Purification of the recombinant protein (r)VvhA

To identify the functional role of VvhA in HCT116 cells, we prepared a recombinant protein of VvhA (rVvhA) from *V. vulnificus*. The oligonucleotides were designed by using the *V. vulnificus* MO6-24/O genomic sequence (GenBank™ accession number CP002469 and CP002470, www.ncbi.nlm.nih.gov) (Park et al., 2011). Briefly, the open reading frame of VvhBA was amplified by performing PCR with a pair of primers for VvhA (Supplementary Table 1) and cloned into a His6 tag expression vector, pET29a (+) (Novagen, Madison, WI, USA) to result in pKS1201 (Table 1). *Escherichia coli* BL21 (DE3) harboring pKS1201 was grown in LB-ampicillin medium at 37 °C until the cultures reached an A_{600} between 0.5 and 0.6. The temperature was then lowered to 30 °C, and protein expression was induced by treatment with 1 mM isopropyl-β-dthiogalactopyranoside (IPTG) for 6 h. The cells were harvested by centrifugation at 5,000 × *g* for 20 min at 4 °C. The cell pellets were resuspended in buffer A (20 mM Tris-Cl, pH 8.0, and 500 mM NaCl), and the cell suspensions were ultrasonicated. The crude cell extracts were centrifuged at 16,000 × *g* for 30 min at 4 °C, and the

supernatant was filtered by using a 0.2 μm Whatman Puradisc syringe filter (Whatman International, Maidstone, Kent, UK) to isolate the soluble fraction. Cell lysate containing His6-tagged VvhBA protein was mixed with 1 ml of nickel-nitrilotriacetic acid agarose (Qiagen, Valencia, CA, USA) for 1 h at 4 °C, and the mixture was loaded on Bio-Spin® chromatography columns (Bio-RadLaboratories, Hercules, CA, USA). The resin was washed with buffer A, and the bound VvhBA protein was eluted with buffer A containing 300 mM imidazole. After purification, the homogeneity of VvhBA was assessed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Purified proteins were dialyzed against 20 mM Tris-Cl, pH 8.0, concentrated to 0.3 mg/ml by using Slide-A-Lyzer dialysiscassettes (Thermo Scientific, Hudson, NH, USA) and stored at −80 °C until use.

2.6. Cell viability

Cell viability assay was performed by using EZ-CYTOX cell viability kit (DaeilLab service, Seoul, Korea) according to the manufacturer's instructions. Cells were cultured on 96-well culture plates. After incubation with rVvhA and RVS glycoprotein, EZ-CYTOX master mix was added to each well. After incubation for 1 h, cell viability was analyzed by measurement absorbance at 450 nm.

2.7. Intracellular reactive oxygen species (ROS) detection

CM-H_2DCFDA was used to detect the intracellular and mitochondrial reactive oxygen species (ROS) production. To quantify the intracellular ROS levels, the cells treated with 10 mM CM-H_2DCFDA 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 (SPARK, Seestrasse, Männedorf, Switzerland) and a fluorescent plate reader at excitation and emission wavelengths of 485 and 535 nm, respectively.

2.8. Western blot analysis

Cells were harvested, washed twice with PBS, and lysed with buffer [20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] for 30 min on ice. Protein concentrations were determined by BCA Protein Assay kits (Pierce, Rockford, IL, USA). Equal amounts of protein (20 μg) were resolved by 8∼12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membranes. The membranes were washed with TBST solution [10 mM Tris-HCl (pH 7.6)], 150 mM NaCl, and 0.05% Tween-20, blocked with 5% skim milk for 30 min and incubated with appropriate primary antibody at 4 °C for overnight. The membrane was then washed twice with TBST solution and incubated with a horseradish peroxidase-conjugated secondary antibody for 2 h. The bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) and detected by using the Bio-Rad Chemi Doc™ XRS + System (Bio-Rad, Hercules, CA, USA). The results of the western blot analysis were calculated in terms of relative intensity, using Scion imaging software (Scion Image Beta 4.02, Frederick, MD, USA).

2.9. Cell number count

To determine total cell numbers, cells were washed twice with PBS and trypsinized from the culture dishes. The cell suspension was mixed with a 0.4% (w/v) trypan blue solution and the number of live cells was determined using a hemocytometer. Cells failing to exclude the dye were considered nonviable.

2.10. Statistical analysis

Results are expressed as means ± standard errors (S.E.). 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 $P < 0.05$.

3. Results

3.1. Inhibitory effect of RVS glycoprotein on cell death induced by V. vulnificus

We previously reported that recombinant protein (r) VvhA at 50 pg/ml has ability to induce apoptotic cell death in gastrointestinal epithelial cells (Lee et al., 2018; Song et al., 2016). To confirm that the VvhA produced by *V. vulnificus* regulates the viability of HCT116 cells, the cells were exposed to rVvhA (50 pg/ml) for various times (0–24 h). The cell viability was determined by EZ-CYTOX reagent containing high-sensitive water-soluble tetrazolium salt reduced by mitochondrial dehydrogenase. rVvhA significantly induced cytotoxicity of the cells from 12 h, compared to the cells with no treatment (Fig. 1A). An increase in cytotoxicity was significantly reversed by co-treatment with glycoprotein isolated from *Rhus verniciflua* stokes (RVS) at concentrations of 50 and 100 μg/ml for 12 h (Fig. 1B). These results demonstrate that biological effect of RVS glycoprotein on *V. vulnificus* infections is related to blockage of cell death induced by rVvhA.

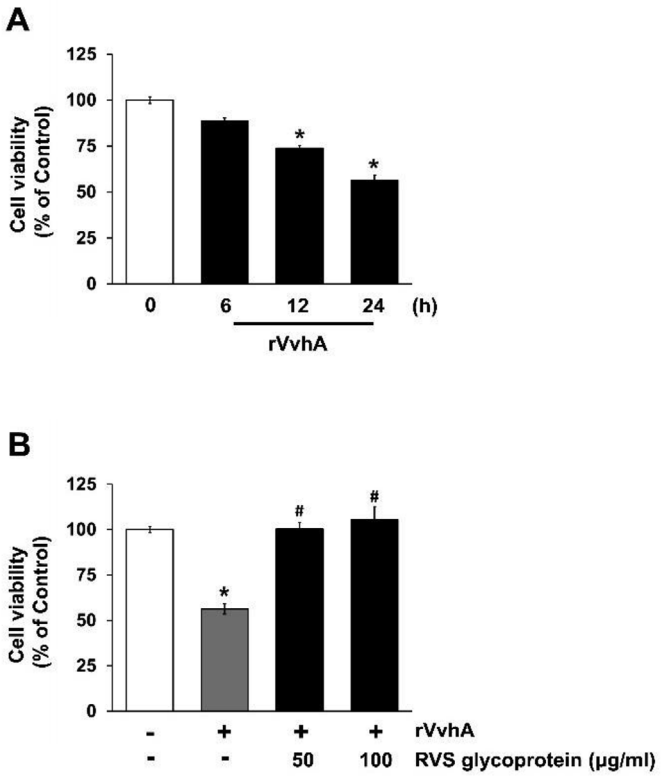

Fig. 1. Inhibitory effect of RVS glycoprotein on cell death induced by *V. vulnificus*. (A) Time responses of cell viability in HCT116 cells treated with 50 pg/ml of rVvhA is shown. Data represent means ± S.E. n = 3. *p ≤ 0.001 versus 0 h. (B) Cells were treated with RVS glycoprotein (50 and 100 μg/ml) in the presence of rVvhA for 12 h. Error bars represent the means ± S.E. n = 3. *p ≤ 0.001 versus the cells with no treatment. #p ≤ 0.01 versus rVvhA alone.

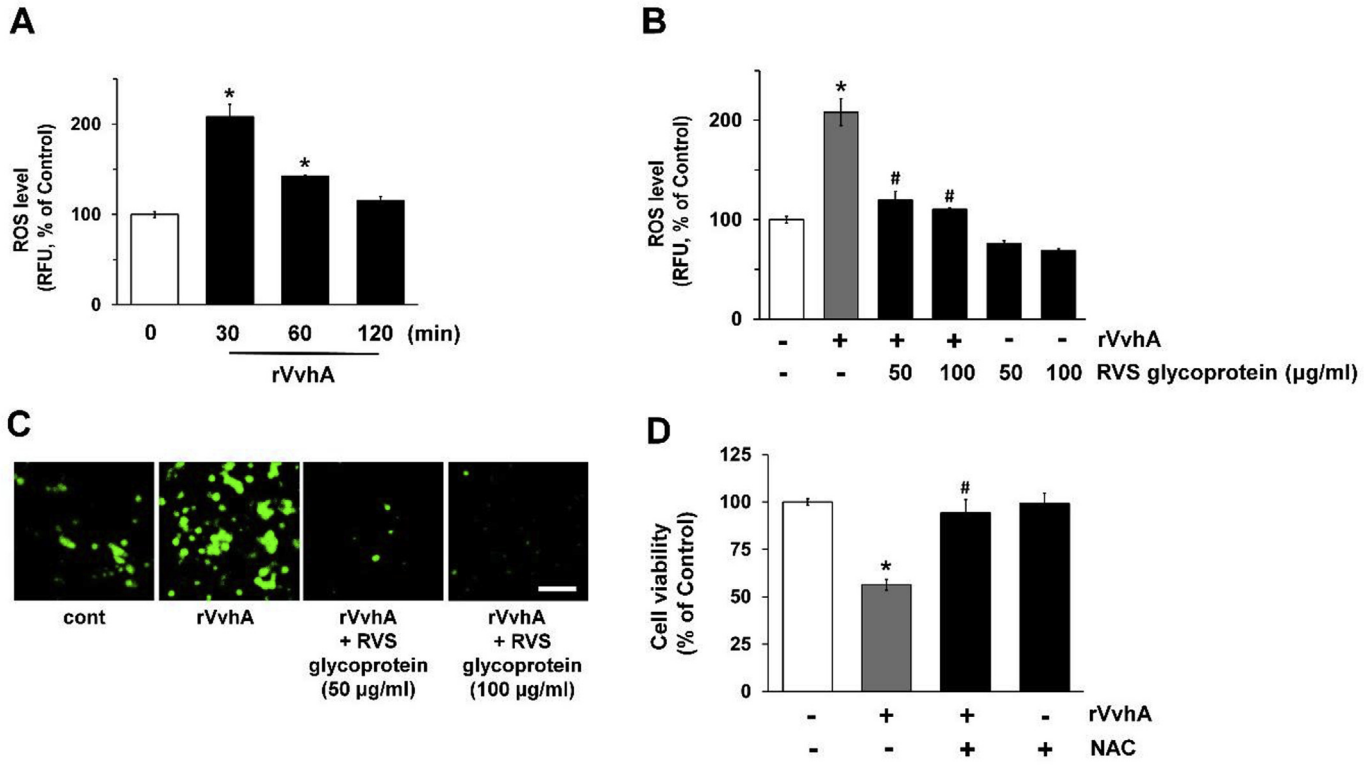

Fig. 2. RVS glycoprotein reduces ROS production to block cell death induced by rVvhA. (A) Time responses of ROS production in cell treated with rVvhA is shown. Data represent the means ± S.E. n = 3. *p ≤ 0.01 versus 0 min. (B) Cells were incubated with RVS glycoprotein for 30 min prior exposure to rVvhA for 30 min. The level of ROS production is shown. Data represent the mean ± S.E. n = 3. *p ≤ 0.001 versus control. #p ≤ 0.01 versus rVvhA alone. (C) ROS production (green) was visualized by confocal microscopy. Scale bars, 100 μm (Original magnification × 400). n = 3. (D) Cells were pretreated with antioxidant, NAC (10 μM) for 30 min prior exposure to rVvhA for 12 h. Cell viability was determined by EZ-CYTOX cell viability kit. n = 3.*p ≤ 0.001 versus the cells with no treatment. #p ≤ 0.01 versus rVvhA alone. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. RVS glycoprotein reduces ROS production to block cell death induced by rVvhA

Since reactive oxygen species (ROS) are critical mediators in the pathophysiology of bacterial infection, we studied the anti-oxidative potentials of RVS glycoprotein in the rVvhA-stimulated HCT116 cells. The production of ROS was measured by using a fluorescent dye, 2′, 7′ -dichlorofluorescein diacetate (CM-H_2DCFDA). The level of ROS production was significantly augmented by treatment with rVvhA after 30 min, compared to the control (Fig. 2A). By contrast, when the cells were co-treated with RVS glycoprotein in presence of the rVvhA, the ROS levels were significantly diminished by 88.3 and 97.9% in ROS production at 50 and 100 μg/ml of RVS glycoprotein, respectively, compared to the rVvhA treatment alone (Fig. 2B). The inhibitory effects of RVS glycoprotein on ROS production were further visualized by staining HCT116 cells with a fluorescent dye, CM-H_2DCFDA (Fig. 2C). To clarify the involvement of ROS production in rVvhA-mediated cytotoxicity, cells were pre-treated with an antioxidant, N-acetylcysteine (NAC). As shown in Fig. 2D, the cytotoxicity induced by rVvhA was significantly blocked by the treatment with NAC. These results indicate that ROS production is required for rVvhA in the promoting of gastrointestinal cell death and that RVS glycoprotein could suppress the cell death induced by *V. vulnificus* via its anti-oxidative potentials.

3.3. RVS glycoprotein inhibits PKC/JNK pathway activated by rVvhA

Protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs) are one of interesting candidates of downstream mediators of ROS in the regulation of host signaling pathway infected with many bacterial pathogens (Lee et al., 2015; Monturiol-Gross et al., 2014; Zhu et al., 2017). The rVvhA treatment significantly induced the phosphorylation of pan-PKC at 30 min and that increase was blocked by treatment with RVS glycoprotein (Fig. 3A) as well as NAC (Fig. 3B). These results indicate that PKC activation is downstream event of ROS production and RVS glycoprotein has ability to suppress the rVvhA signaling pathway related to phosphorylation of PKC. Importantly, blockage of PKC with PKC inhibitor, Bisindolylmaleimide I significantly abrogated the cell death induced by rVvhA, suggesting that a functional role of PKC in regulating rVvhA-mediated cytotoxicity (Fig. 3C). On the other hand, the rVvhA treatment also increased the phosphorylation of JNK and ERK, but it did not affect the phosphorylation of p38 MAPK (Fig. 3D). Moreover, the effect of rVvhA on activation of JNK and ERK at 30 min could be inhibited by treatments of RVS glycoprotein (Fig. 3D) as well as Bisindolylmaleimide I (Fig. 3E). These data indicate that phosphorylation of JNK and ERK mediated by PKC plays key role in the promotion of cell death induced by rVvhA and that rVvhA signaling pathway can be negatively regulated by RVS glycoprotein. Interestingly, the increase in cytotoxicity induced by rVvhA was significantly reversed by treatment with JNK inhibitor, SP600125 (Fig. 3F). Despite the significant role of rVvhA in ERK activation, the ERK inhibitor, PD98059 did not affect rVvhA-induced cell death, suggesting that the signaling events related to ERK are unrelated to the cytotoxicity induced by rVvhA (Fig. 3F).

3.4. Effect of RVS glycoprotein on activation of NF-κB induced by rVvhA

We further examined the effect of RVS glycoprotein on the activation of NF-κB, which is a direct transcriptional target for cell death pathway mediated by ROS. As shown in Fig. 4A, the phosphorylation of NF-κB was up-regulated after treatment with rVvhA for 30 min, compared to the control. However, the activation of NF-κB was significantly inhibited by treatments with RVS glycoprotein (Fig. 4A) and JNK

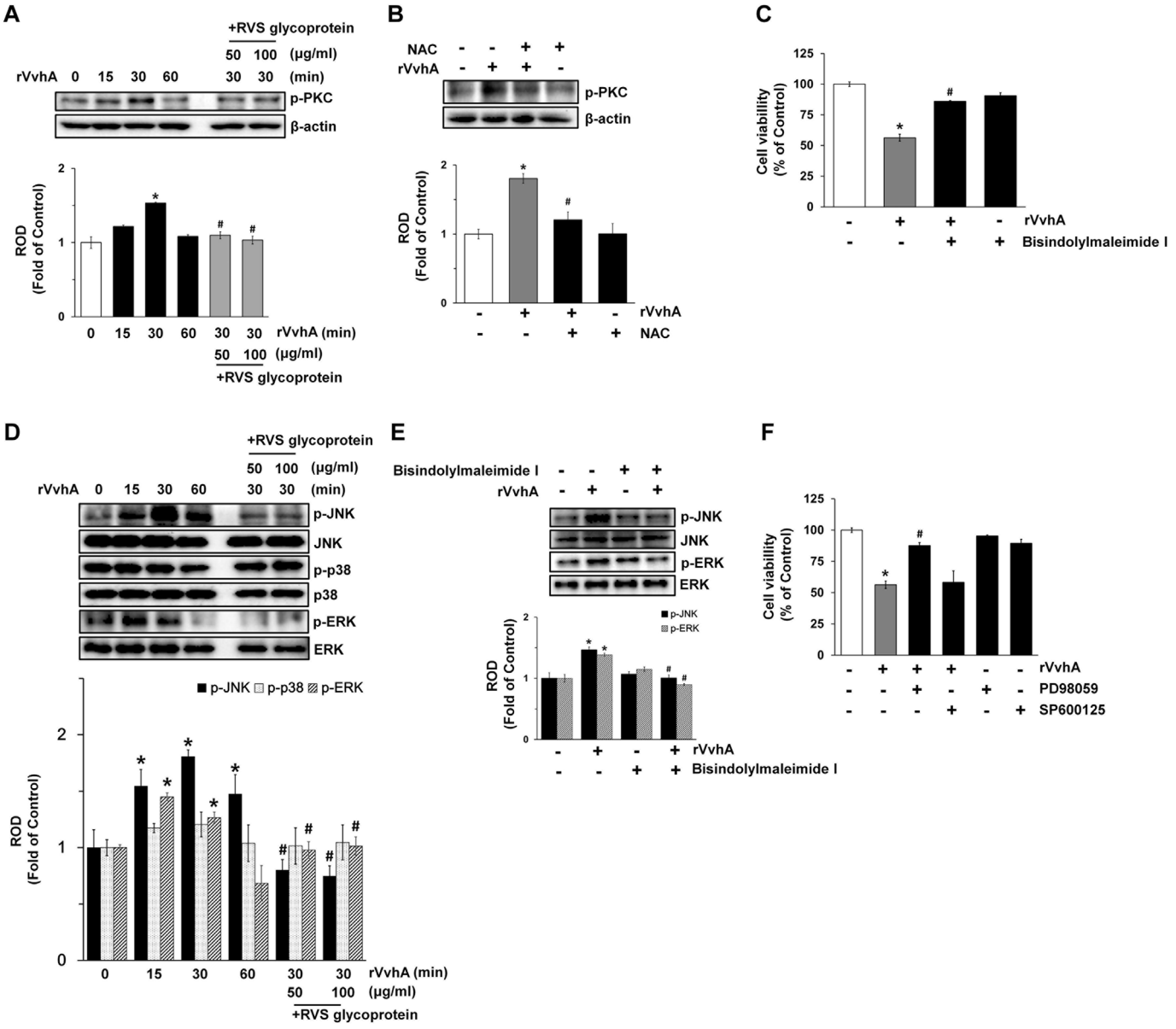

Fig. 3. RVS glycoprotein inhibits PKC/JNK pathway activated by rVvhA. (A) Time responses of phosphorylation of pan-PKC in cell co-treated with RVS glycoprotein and rVvhA are shown. Data represent the mean ± S.E. n = 3. *p ≤ 0.001 versus control. #p ≤ 0.001 versus rVvhA alone. (B) Cells were pretreated with NAC (10 μM) for 30 min prior exposure to rVvhA for 30 min. Phosphorylation of PKC is shown. Data represent the mean ± S.E. n = 3. *p ≤ 0.01 versus control. #p ≤ 0.05 versus rVvhA alone. (C) Cells pretreated with PKC inhibitor, Bisindolylmaleimide I (10 μM) were incubated with rVvhA for 12 h. Cell viability was determined by EZ-CYTOX cell viability kit. Data represent the mean ± S.E. n = 3. *p ≤ 0.001 versus control. #p ≤ 0.001 versus rVvhA alone. (D) The effect of RVS glycoprotein on the phosphorylation of MAPK. Time responses of phosphorylation of MAPK in cell co-treated with RVS glycoprotein and rVvhA are shown. Data represent the mean ± S.E. n = 3. *p ≤ 0.05 versus control. #p ≤ 0.01 versus rVvhA alone. (E) Cells pretreated with Bisindolylmaleimide I were incubated with rVvhA for 30 min. The level of phosphorylation of JNK and ERK are shown. Data represent the mean ± S.E. n = 3. *p < 0.01 versus control. #p ≤ 0.05 versus rVvhA alone. (F) Cells pretreated with JNK inhibitor, SP600125 (10 μM) and ERK inhibitor, PD98059 (10 μM) were incubated with rVvhA for 12 h. Cell viability was determined by EZ-CYTOX cell viability kit. Data represent the mean ± S.E. n = 3. *p ≤ 0.001 versus control. #p ≤ 0.001 versus rVvhA alone. ROD, relative optical density.

inhibitor, SP600125 (Fig. 4B). These results indicate that NF-κB activation is downstream event of JNK activation and RVS glycoprotein blocks the rVvhA signaling pathway related to transcriptional regulation of NF-κB. In addition, cell death induced by rVvhA was significantly blocked by the NF-κB inhibitor, Bay 11–7082 (Fig. 4C), suggesting that the activity of NF-κB is necessary for cytotoxicity induced by rVvhA. Taken together, the above results indicate that RVS glycoprotein is a functional substance that regulates transcriptional activity of NF-κB mediated by JNK in rVvhA-stimulated HCT116 cells.

3.5. RVS glycoprotein inhibits expression of Bax and cleaved-caspase 3 induced by rVvhA

We further determined whether RVS glycoprotein has inhibitory effect on expression of cell death targets that are related to the NF-κB activation for apoptotic cell death. HCT116 cells were exposed to RVS glycoprotein in the presence of rVvhA for 12 h. The exposure of rVvhA increased Bcl-2 associated X protein (Bax) expression (Fig. 5A) but decreased Bcl-2 expression (Fig. 5B), suggesting that rVvhA induces mitochondrial apoptotic cell death in a time-dependent manner. Consistent with these results, rVvhA stimulated the expression of caspase-3

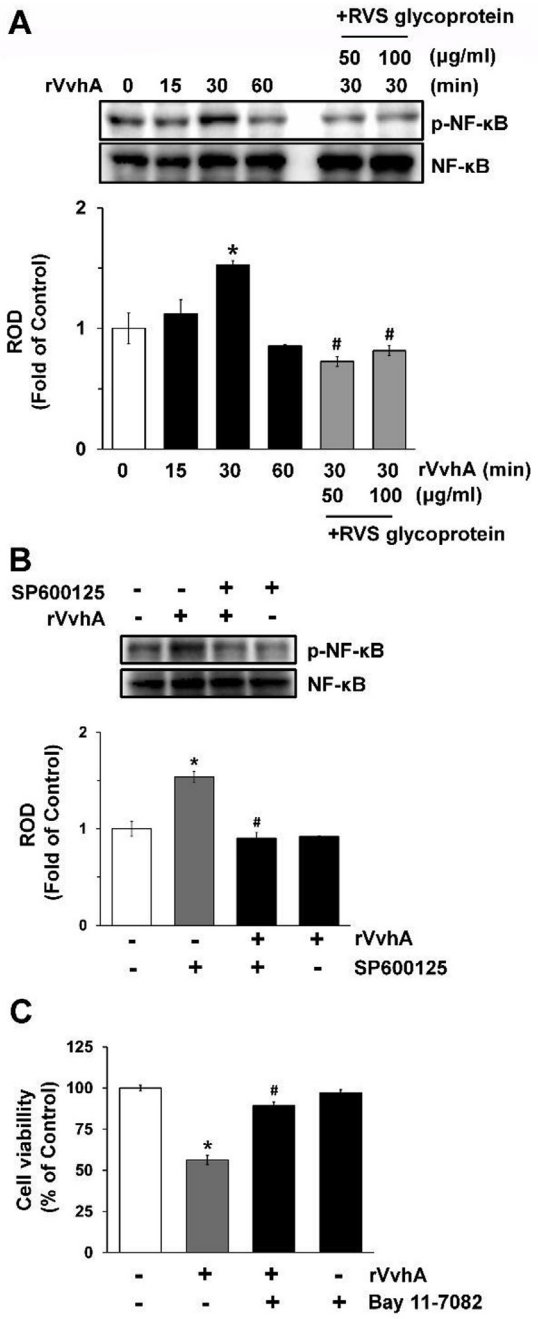

Fig. 4. Effect of RVS glycoprotein on activation of NF-κB induced by rVvhA. (A) Time responses of phosphorylation of NF-κB in cell co-treated with RVS glycoprotein and rVvhA are shown. Data represent the mean ± S.E. n = 3. *p ≤ 0.01 versus control. #p ≤ 0.001 versus rVvhA alone. (B) Cells pretreated with SP600125 were incubated with rVvhA for 30 min. The level of NF-κB phosphorylation was determined by western blot. Data represent the mean ± S.E. n = 3. *p ≤ 0.01 versus control. #p ≤ 0.001 versus rVvhA alone. (C) Cells were pretreated with NF-κB inhibitor, Bay 11–7082 (10 μM) for 30 min prior exposure to rVvhA for 12 h. Cell viability was determined by EZ-CYTOX cell viability kit. Data represent the mean ± S.E. n = 3. *p ≤ 0.001 versus control. #p ≤ 0.001 versus rVvhA alone.

cleavages responsible for apoptosis execution (Fig. 5C). However, treatment with RVS glycoprotein as well as NF-κB inhibitor, Bay 11–7082 significantly blocked the increase in Bax, decrease in Bcl-2 and increase in cleaved-caspase 3, which are all induced by rVvhA (Fig. 5A–D). These results mean that the involvement of NF-κB at a key step in mitochondrial apoptosis during rVvhA exposure and demonstrate that RVS glycoprotein inhibits cell death by regulating expression of mitochondrial apoptotic genes in rVvhA-treated HCT116 cells. To confirm that the functional effect of RVS glycoprotein to manipulate bacterial signaling pathway is mediated through its related signaling molecules proved by our study, we further determined the effect of inhibitors on activation of rVvhA signaling pathway. We pretreated cells with NAC, Bisindolylmaleimide I, SP600125 and Bay 11–7082 to confirm involvement of ROS, PKC, JNK and NF-κB for 30 min prior to rVvhA exposure for 12 h (Fig. 5E). As expected, the rVvhA-induced decrease in cell number was significantly abrogated by RVS glycoprotein as well as all blockers for ROS, PKC, JNK and NF-κB, respectively.

4. Discussion

In this study we demonstrate that a 36 kDa plant glycoprotein isolated from RVS can block apoptosis induced by *V. vulnificus* VvhA via the inhibition of ROS-dependent PKC/MAPK/NF-κB activation in gastrointestinal epithelial cells. It was previously shown that hemolytic toxin VvhA together with cytotoxin RTX significantly contribute to the pathogenesis of *V. vulnificus* and induce intestinal tissue damage and inflammation that lead to the dissemination of the infecting bacteria to the bloodstream and other organs (Jeong and Satchell, 2012). These results are supported further by our previous results showing that VvhA not only acts as a hemolysin but that it has the ability to induce mitochondrial apoptotic cell death in gastrointestinal epithelial cells (Lee et al., 2018). Given that VvhA plays a critical role in the promotion of multiple cytotoxic effects on host cells, it is important to find pharmacological substances that regulate the toxicological signaling cascades which occur during *V. vulnificus* infections. In recent years, many scientists have insisted that phytochemicals in fruits and vegetables have functional roles in preventing many diseases caused by reactive oxidants, such as superoxide anions, hydrogen peroxide, and NADPH oxidase (Hirota et al., 2002; Ko et al., 2006). Many functional glycoproteins have been isolated from mushrooms, fungi, yeasts, algae, lichens, and plants due to the many biological activities stemming from their anti-oxidative, immunomodulatory, and anti-carcinogenic properties based on their high structural variability and polarity (Ooi and Liu, 2000). However, the underlying pharmacological mechanisms of these substances against *V. vulnificus* infection in gastrointestinal epithelial cells have not been reported.

In the present study, we present new findings revealing that RVS glycoprotein has the ability to inhibit the apoptotic mechanism induced by VvhA. VvhA is an important toxin of *V. vulnificus* that possibly forms a redox signaling platform within lipid rafts of the host cell membrane to amplify a variety of ROS-dependent signaling pathways during the regulation of apoptosis (Larrick and Wright, 1990). The infection of bacteria into host cells causes significant damage to a variety of intracellular macromolecules via ROS production, resulting in mutations of nucleic acids and often in cell death (Trachootham et al., 2009). Thus, it has been suggested ROS are significant cytotoxic and signaling mediators in the pathophysiology of bacterial infection (Fang, 2011). These results are consistent with our results showing that apoptotic and autophagic cell death induced by *V. vulnificus* VvhA can be inhibited by a well-known antioxidant, in this case melatonin (Lee et al., 2018). Moreover, we demonstrated that RVS glycoprotein acts as a functional natural antioxidant against cytotoxicity and apoptosis which are induced by hydroxyl radicals and superoxide anions (Ko et al., 2005). Therefore, our results in the present study indicate that RVS glycoprotein has the ability to block cell death induced by rVvhA via its antioxidant capacity to scavenge intracellular ROS production in HCT116 cells.

The PKC and MAPK pathways, which are interesting candidates as downstream mediators of ROS, are modulated by many bacterial stimuli. Given that ROS are major modulators during the activation of the PKC signaling pathway via the regulation of phospholipase C (Wu et al.,

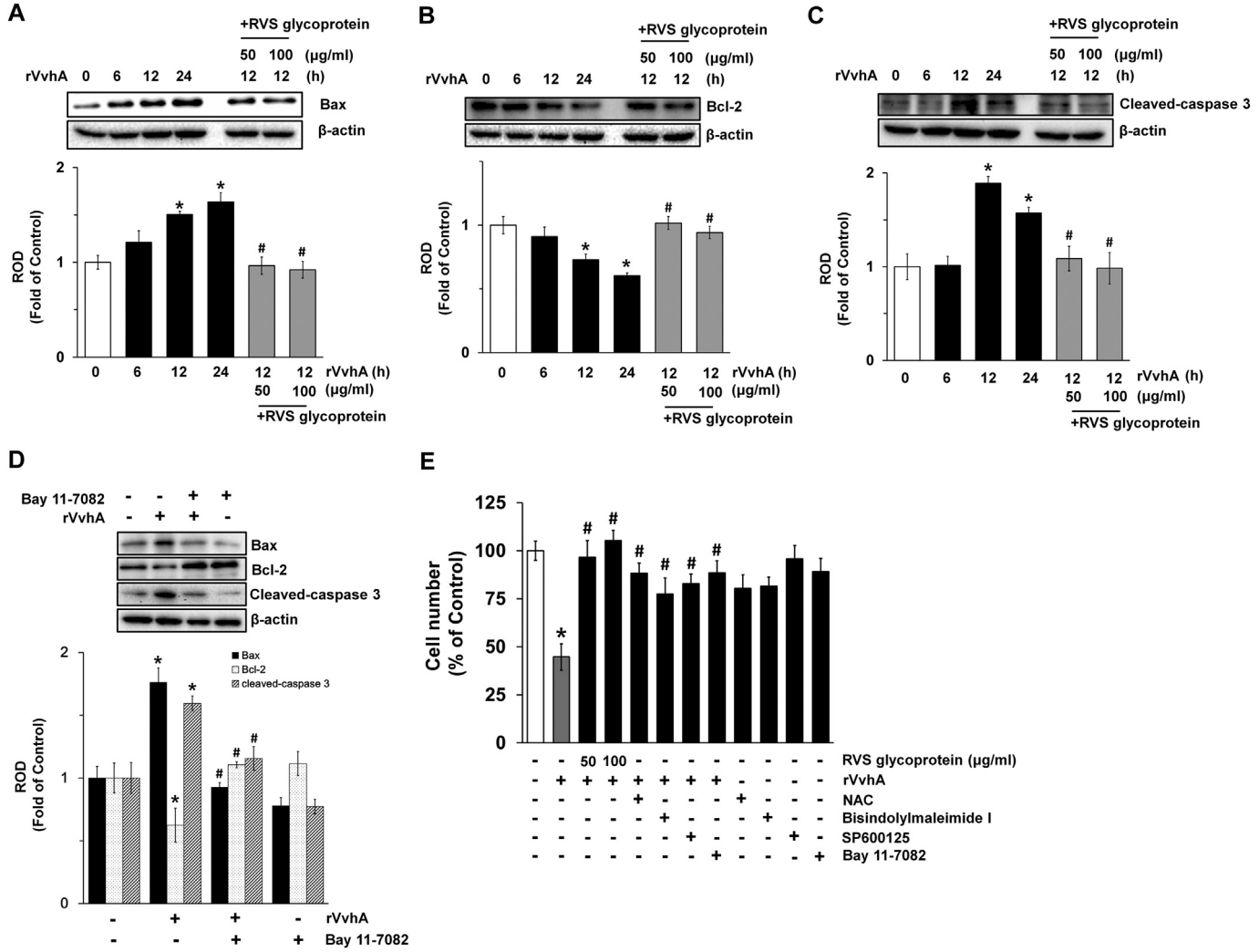

Fig. 5. RVS glycoprotein inhibits expression of Bax and cleaved-caspase 3 induced by rVvhA. (A) Time responses of expression of Bax in cell co-treated with RVS glycoprotein and rVvhA are shown. Data represent the mean ± S.E. n = 3. *p ≤ 0.05 versus control. #p ≤ 0.05 versus rVvhA alone. (B) Time responses of expression of Bcl-2 in cell co-treated with RVS glycoprotein and rVvhA are shown. Data represent the mean ± S.E. n = 3. *p ≤ 0.01 versus control. #p ≤ 0.05 versus rVvhA alone. (C) Time responses of expressions cleaved-caspase 3 in cell co-treated with RVS glycoprotein and rVvhA are shown. Data represent the mean ± S.E. n = 3. *p ≤ 0.01 versus control. #p ≤ 0.05 versus rVvhA alone. (D) Cells pretreated with Bay 11–7082 for 30 min and incubated with rVvhA for 12 h. The expressions of Bax, Bcl-2 and cleaved-caspase 3 were determined by western blot. Data represent the mean ± S.E. n = 3. *p ≤ 0.01 versus control. #p ≤ 0.05 versus rVvhA alone. (E) Cells were pretreated with NAC, Bisindolylmaleimide I, SP600125 and Bay 11–7082 for 30 min and then exposure to RVS glycoprotein and rVvhA for 12 h. The number of cell was determined by cell counting assay. Data represent the mean ± S.E. n = 4.*p ≤ 0.001 versus control. #p ≤ 0.01 versus rVvhA alone.

2008), our results here indicate that RVS glycoprotein inhibits PKC activation stimulated by ROS to attenuate the apoptotic signaling pathway in rVvhA-treated HCT116 cells. These results are supported further by previous findings showing that PKC plays a critical role in promoting the cell death process induced by ROS during EPEC, *Clostridium perfringens,* and *V. vulnificus* infections (Ki et al., 2008; Lee et al., 2018; Nougayrede and Donnenberg, 2004). It has also reported that PKC influences the activation of MAPKs during apoptotic cell death process (Chen et al., 2002). We previously demonstrated that the phosphorylation of JNK is significantly modulated by PKC during the promotion of apoptotic cell death induced by rVvhA (Lee et al., 2018). Thus, our results indicate that the inhibitory role of RVS glycoprotein on the phosphorylation of JNK is critical for the suppression of the apoptotic signaling pathway induced by rVvhA. Moreover, it was previously determined that a bacterial lipopolysaccharide (LPS) treatment results in the activation of JNK in macrophages (Hambleton et al., 1996). Hence, JNK inactivation or silencing by food phytochemicals may be effective against cell death facilitated by a bacterial infection, suggesting that JNK is one of the major regulators and a potential target for apoptosis induced by rVvhA. Consequently, our results suggest that RVS glycoprotein has a curative effect on apoptotic cell death process as caused by *V. vulnificus* infection via the suppression of JNK activation.

Our results in the present study also showed that rVvhA can induce the phosphorylation of NF-κB through JNK and that the inhibition of NF-κB by RVS glycoprotein blocks rVvhA-induced apoptotic cell death (Hambleton et al., 1996). 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 (Ashida et al., 2011). NF-κB transcriptionally regulates the expression levels of many pro-inflammatory mediators that orchestrate and sustain the inflammatory response and causes tissue damage (Lawrence, 2009). Indeed, several studies have found that bacteria or bacterial products, such as *Streptococcus*, *Bacillaceae*, *Campylobacter* and *V. vulnificus* activate NF-κB during the regulation of many cellular responses (Lee et al., 2015; Mellits et al., 2002; Miettinen et al., 2000). Regarding the role of MAPKs in NF-κB activation, earlier work showed that the JNK pathway

induced by ROS can influence NF-κB activation in promoting apoptotic cell death (Lindsay et al., 2011). Hence, it is possible that ROS stimulated by rVvhA has a potential role in promoting the NF-κB pathway via the activation of JNK. Based on these results, we suggest that RVS glycoprotein exerts anti-oxidative effects on NF-κB-dependent signaling pathways during the suppression of apoptotic cell death induced by *V. vulnificus*.

Interestingly, the present results indicate that RVS glycoprotein is able to regulate a shift in the Bax/Bcl-2 ratio via the inhibition of NF-κB activity in rVvhA-treated HCT116 cells. Studies have indicated that the transcriptional activation of NF-κB by many bacterial pathogens influences on the expressions of Bcl-2 family involved in the regulation of mitochondrial cell death pathway (Brunelle and Letai, 2009). The ratio of Bax to Bcl-2 has been suggested as a primary determining factor of the degree of susceptibility to the expression of cleaved–caspase 3 (Yang et al., 2002). In addition, Bax is known to have a specific promoter region for binding NF-κB during the promotion of mitochondrial cell death (Grimm et al., 2005; Wang et al., 2002). These results indicate that the inhibitory effects of RVS glycoprotein on Bax expression as evoked by rVvhA may suppress mitochondrial apoptotic cell death against bacterial infections.

Collectively, this study demonstrates the roles of a phytoglycoprotein during the regulation of the infectious mechanisms of *V. vulnificus* VvhA. In conclusion, RVS glycoprotein inhibits ROS-mediated PKC/MAPK/NF-κB pathway, which leads to the abrogation of rVvhA-induced mitochondrial apoptotic cell death. These results offer important insight into the potential for the development of therapeutic strategies and agents for *V. vulnificus* infections.

Conflicts of interest

The authors have declared that there is no conflict of interest.

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Appendix A. Supplementary data

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