Inhibitory effect of withaferin A on *Helicobacter pylori*-induced IL-8 production and NF-κB activation in gastric epithelial cells

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Abstract. Withaferin A (WA), a withanolide purified from *Withania somnifera*, has been known to exert anti-inflammatory effects. The present study sought to determine the effects of WA on *Helicobacter* (*H.*) *pylori*-mediated inflammation in the AGS gastric epithelial cell line. Cellular production of interleukin (IL)-8 and vascular endothelial growth factor (VEGF) was measured by ELISA. Western blot analysis was performed to determine the activation of nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs) as well as hypoxia-inducible factor 1α stabilization. Bacterial growth was also examined by measuring the optical density. Pre-treatment or co-treatment with WA efficiently reduced IL-8 production by AGS cells in response to *H. pylori* infection. *H. pylori*-induced activation of NF-κB, but not MAPKs, was also inhibited by pre-treatment of WA in the cells. However, WA did not affect VEGF production and HIF-1α stabilization induced by *H. pylori* in AGS cells. In addition, WA did not influence the growth of *H. pylori*, suggesting that the anti-inflammatory effect of WA was not due to any bactericidal effect. These findings indicate that WA is a potential preventive or therapeutic agent for *H. pylori*-mediated gastric inflammation.

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Introduction

Withania somnifera has been applied for the treatment of chronic diseases in Indian Ayurvedic medicine and its therapeutic effects are attributed to steroidal lactones referred to as withanolides. Among these, withaferin A (WA) is known to have anti-inflammatory and anti-cancer properties (1-4). WA inhibits the expression of inducible nitric oxide synthase as well as nitric oxide (NO) production in lipopolysaccharide (LPS)-treated macrophages by downregulating AKT and activating nuclear factor (NF)-κB (5). It also exerts inhibitory effects on high mobility group box 1-induced NF-κB activation and production of interleukin (IL)-6 as well as tumor necrosis factor (TNF)-α in human umbilical vein endothelial cells (6). In addition, WA inhibits constitutive or induced expression of inflammatory mediators, including cytokines and intercellular or vascular adhesion molecules in various types of cell, including epithelial cells (1-4), suggesting that WA is able to exert its anti-inflammatory effects in a wide range of host cells. In addition, WA exhibits marked anti-tumor activity against multiple types of tumor cell, including leukemia (7) as well as prostate (8) and lung (9) cancer cells. Induction of apoptosis and inhibition of DNA synthesis have been suggested as the underlying mechanisms of the anti-proliferative effects of WA on multiple tumor types (10,11); however, the exact mechanisms remain to be elucidated.

Gastric cancer is the fourth most common cancer type worldwide and the third leading cause of mortality from cancer (12,13). Among the various risk factors, including gender, age or diet, *Helicobacter* (*H.*) *pylori* infection is the best-known risk factor for gastric adenocarcinoma and has been estimated to account for 60% of gastric cancer cases worldwide (14,15). The inflammatory response induced by *H. pylori* infection is considered to be a major step in the initiation and development of gastric cancer (16).

Reducing inflammation induced by *H. pylori* infection may be an effective means of preventing and curing gastric cancer.
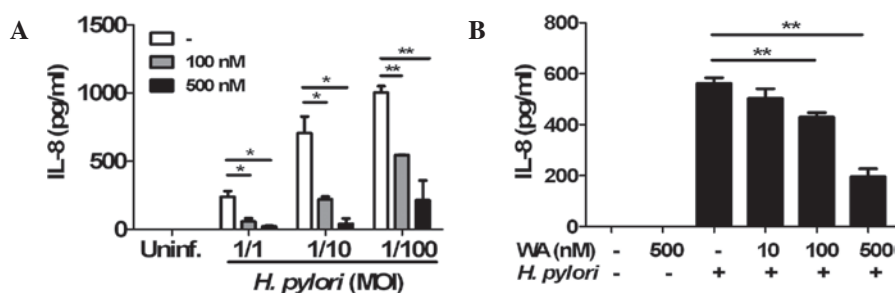
Figure 1. WA inhibits *H. pylori*-induced IL-8 production in gastric epithelial cells. (A) ASG cells were pre-treated with or without WA for 6 h, followed by infection with the indicated MOI of *H. pylori* for 24 h. (B) In another experiment (co-treatment), the cells were also infected with *H. pylori* (MOI, 1/100) in the absence or presence of WA. IL-8 levels in the culture supernatants was measured by ELISA. Values are expressed as the mean ± standard deviation of triplicate samples from one experiment representative of three independent experiments. *P<0.5, **P<0.01, ***P<0.001. *H. pylori*, *Helicobacter pylori*; MOI, multiplicity of infection; IL interleukin; WA, withaferin A; Uninf., uninfected.

It is therefore of great global interest to discover novel preventive and therapeutic agents from a pool of natural products with activity against inflammatory diseases and cancer (9,17-19). Although WA possesses anti-inflammatory as well as anti-cancer properties against a broad range of cell types, its efficacy against gastric inflammation and cancer has not yet been evaluated, to the best of our knowledge. Therefore, the present study assessed the inhibitory effects of WA on *H. pylori*-induced production of IL-8 and vascular endothelial growth factor (VEGF), which are key inflammatory mediators associated with tumor progression (20-24), as well as the mitogen-activated protein kinase (MAPK) pathway. In addition, the inhibitory effect of WA on the proliferation of gastric cancer cells was assessed and the underlying molecular mechanisms were investigated.

Materials and methods

H. pylori strain and culture conditions. The *H. pylori* strain 26695 (American Type Culture Collection, Manassas, VA, USA) was grown on campylobacter agar (BD Biosciences, Franklin Lakes, NJ, USA) or brucella broth (BD Biosciences) containing 10% fetal bovine serum (FBS; Corning Incorporated, Corning, NY, USA), 10 μg/ml vancomycin (Sigma-Aldrich, St. Louis, MO, USA), 5 μg/ml trimethoprim (Sigma-Aldrich), and 1 μg/ml nystatin (Sigma-Aldrich) at 37°C under microaerobic conditions. The bacteria were grown to an optical density at 600 nm (OD_{600}) of 0.6, measured using an enzyme-linked immunosorbent assay (ELISA) reader (Epoch; Bio-Tek Instruments, Inc., Winooski, VT, USA), which corresponds to ~10^9 colony-forming units (CFU)/ml, and diluted to the desired concentrations (16).

Cell culture and treatment. The AGS human gastric epithelial cell line was purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured with RPMI-1640 medium (Welgene, Inc., Daegu, Korea) containing 10% FBS and 1X penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified atmosphere containing 5% CO_2 at 37°C. To determine the production of VEGF and IL-8, AGS cells ($1x10^5$ cells/well in a 48-well plate) were infected with *H. pylori* 26695 at the indicated multiplicity of infection (MOI; 1, 10, 50 or 100) in the absence or presence of WA (10-500 nM; Sigma-Aldrich) for 24 h at 37°C in an atmosphere containing 5% CO_2. To evaluate the levels of

hypoxia-inducible factor (HIF)-1α, AGS cells were infected with *H. pylori* 26695 at an MOI of 100 with or without WA (500 nM) for 6 h.

Determination of IL-8 and VEGF. The concentration of IL-8 and VEGF in the culture supernatants of *H. pylori*-infected AGS cells was determined by commercial Duoset ELISA kits (cat no. DY208 for IL-8 and cat no. DY293B for VEGF; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The MTT)-based assay was performed to determine the cytotoxicity of WA on AGS cells. The cells were seeded at a density of $5x10^5$ cells/well in 48-well plates with growth medium. After 24 h, the cells were exposed to different concentrations of WA (0, 10, 25, 50, 100, 250, 500 and 1000 nM). After 24 h, each well was incubated with MTT (4 mg/ml; Sigma-Aldrich) in RPMI-1640 medium (Welgene, Inc.) for 4 h at 37°C. After 4 h, the MTT solution was removed and replaced with 200 μl of dimethyl sulfoxide (Sigma-Aldrich). The plates were shaken for 5 min to dissolve the MTT formazan crystals. The OD of each well was determined using an ELISA reader (Epoch) at a wavelength of 570 nm. Experiments were repeated in triplicate, and 6 parallel samples were measured each time.

Immunoblotting. AGS cells were infected with *H. pylori* 26695 (MOI, 100) with or without pre-treatment with WA (500 nM) for 6 h and lysed at the indicated time-points (0, 15, 30 or 60 min) in a buffer containing 1% Nonidet-P40 supplemented with protease inhibitor (complete Mini EDTA-free; Roche, Mannheim, Germany), phosphatase inhibitor (Phosphatase Inhibitor Cocktail 2; Sigma-Aldrich) and 2 mM dithiothreitol (Sigma-Aldrich). The extracted protein concentration was measured using a Protein Assay kit (cat no. 500-0006; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples of protein (30 μg) were cooled on ice following an incubation at 95-100°C for 15 min, and the samples were subsequently electrophoresed using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For the electrophoresis, stacking gel and separating gel were used at a constant voltage (100 V) for 90 min and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.) by electroblotting. For the electrotransfer, the apparatus
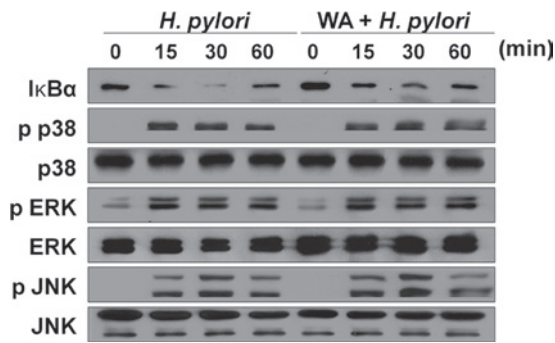
Figure 2. WA inhibits the activation of NF-κB, but not MAPKs, induced by *H. pylori* infection in gastric epithelial cells. AGS cells were pre-treated with or without 500 nM of WA and subsequently infected with *H. pylori* (multiplicity of infection, 1/100). The cellular proteins were harvested at the indicated time-points and the amounts of phosphorylated or total MAPKs (p38, ERK or JNK) and IκB-α were determined by western blot analysis. MAPK, mitogen-activated protein kinase; p-ERK, phosphorylated extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappa B; IκBα, inhibitor of NF-κB; *H. pylori, Helicobacter pylori*; WA, withaferin A.
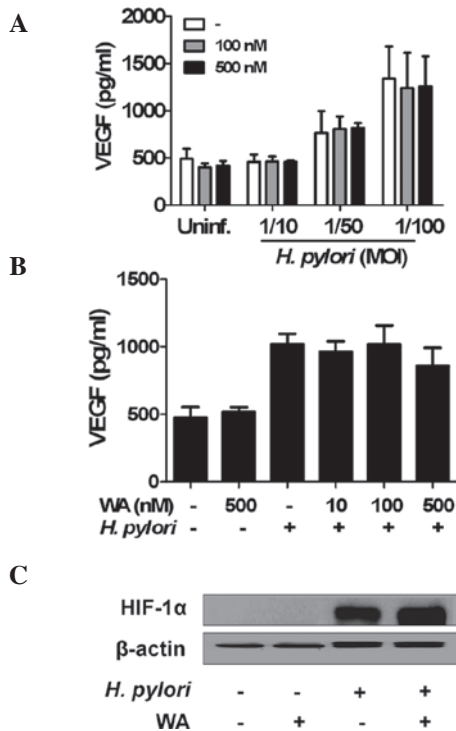

Figure 3. WA does not affect *H. pylori*-induced VEGF production and HIF-1α stabilization in gastric epithelial cells. (A) ASG cells were pre-treated with or without WA for 6 h, followed by infection with the indicated MOI of *H. pylori* for 24 h. (B) In another experiment (co-treatment), the cells were also infected with *H. pylori* (MOI, 1/100) in the absence or presence of WA. At 24 h after infection, the concentration of VEGF in culture supernatant was determined by ELISA. Values are expressed as the mean ± standard deviation of triplicate samples from one experiment representative of three independent experiments. (C) The cells were pre-treated with WA (500 nM) and infected with *H. pylori* at an MOI of 100 for 6 h. Cellular protein was extracted and the levels of HIF-1α were analyzed by immunoblotting. β-actin was used as a loading control. VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; *H. pylori, Helicobacter pylori*; MOI, multiplicity of infection; IL interleukin; WA, withaferin A; Uninf., uninfected.

was powered by a constant current (100 V) for 2 h. The nitrocellulose membranes were blocked with blocking buffer of
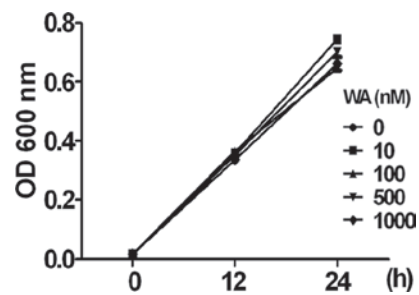

Figure 4. WA does not exhibit any anti-bacterial activity against *H. pylori*. *H. pylori* was cultured in brucella broth containing various doses of WA at 37°C under microaerobic conditions for 12 and 24 h. Bacterial growth was determined by measuring the OD at 600 nm. OD, optical density; *H. pylori, Helicobacter pylori*; WA, withaferin A.

5% skimmed milk (incubated at room temperature for 1 h), and subsequently the blocking solution was discarded. The membranes were immunoblotted with primary antibodies as follows: rabbit anti-human polyclonal IκB-α (1:1000; cat. no. 9242; Cell Signaling Technology, Inc., Danvers, MA, USA); rabbit anti-human polyclonal phosphorylated (p)-c-Jun N-terminal kinase (JNK; 1:1000; cat. no. 9251; Cell Signaling Technology, Inc.); rabbit anti-human polyclonal JNK antibody (1:1000; cat. no. 9252; Cell Signaling Technology, Inc.); rabbit anti-human polyclonal p-p38 antibody (1:1000; cat. no. sc-101759; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); rabbit anti-human polyclonal p38 antibody (1:1000; cat. no. sc-728; Santa Cruz Biotechnology, Inc.); mouse anti-human monoclonal p-ERK antibody (1:1000; cat. no. sc-7383; Santa Cruz Biotechnology, Inc.); rabbit anti-human polyclonal ERK antibody (1:1000; cat. no. sc-94; Santa Cruz Biotechnology, Inc.); mouse anti-human monoclonal HIF-1α antibody (1:1000; cat. no. 610958; BD Biosciences); and rabbit anti-human polyclonal anti-β-actin antibody (1:1000; cat. no. sc-130656; Santa Cruz Biotechnology, Inc.) were added, followed by an incubation with agitation at 4°C overnight. The membrane was rinsed with Tris-buffered saline with Tween® 20 (TBST) three times, each time for 5 min. The membrane was incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit (1:4000; cat. no. sc-2301; Santa Cruz Biotechnology, Inc.) or goat anti-mouse IgG (1:2000; cat. no. sc-2031; Santa Cruz Biotechnology, Inc.) antibodies for 2 h at room temperature, and again washed three time with TBST for 10 min. Proteins were detected with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). Images of the blots were captured on CP-BU new film (Agfa HealthCare, Mortsel, Belgium) by an Automatic X-ray Film processor (JP-33; JPI, Seoul, Korea).

Bacterial growth rate. 50 μl of the bacterial cultures (1×10^9 CFU/ml) were diluted with 2 ml brucella broth containing WA (10-1,000 nM) and incubated at 37°C under microaerobic conditions for 12 and 24 h. Bacterial growth was determined by measuring the OD_{600} of the culture broth.

Statistical analysis. Values are expressed as the mean ± standard deviation. Differences between mean values among different groups were tested and all statistical calculations were performed by one- or two-way analysis of

variance with Bonferroni's post-hoc test using GraphPad Prism version 5.00 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference between values.

Results

WA inhibits H. pylori-induced IL-8 production in gastric epithelial cells. To determine the effects of WA on *H. pylori*-induced IL-8 production in gastric epithelial cells, AGS cells were pre-treated with 100 or 500 nM WA for 6 h and subsequently infected with *H. pylori*. An ELISA showed that the two doses of WA significantly reduced IL-8 production induced by *H. pylori* infection (Fig. 1A). In a further experiment, the cells were co-treated with *H. pylori* (MOI, 1/100) and various doses of WA. Following incubation for 24 h, *H. pylori*-induced production of IL-8 was decreased by WA in a dose-dependent manner (Fig. 1B). Furthermore, a preliminary MTT assay revealed that WA was not cytotoxic at the concentrations used (data not shown). These results indicated that pre-treatment and co-treatment with WA effectively inhibited *H. pylori*-induced IL-8 production in gastric epithelial cells.

WA inhibits the activation of NF-κB, but not MAPKs, induced by H. pylori infection in gastric epithelial cells. NF-κB and MAPKs are known to be involved in *H. pylori*-induced IL-8 production in gastric epithelial cells (25). Therefore, the present study sought to determine whether WA affects the activation of NF-κB and MAPKs in *H. pylori*-infected gastric epithelial cells. Infection of AGS cells with *H. pylori* for 15 min led to a marked degradation of IκB-α, which almost disappeared at 30 min (Fig. 2). Of note, this reduction of IκB-α was restored by WA treatment (Fig. 2). By contrast, the MAPKs p38, ERK and JNK were activated by *H. pylori* at 15 min of infection and beyond, which was not affected by treatment with WA. These results indicated that WA specifically inhibits the activation of NF-κB induced by *H. pylori* in gastric epithelial cells, while not affecting the activation of MAPKs,.

WA does not affect H. pylori-induced VEGF production and HIF-1α stabilization in gastric epithelial cells. To evaluate the effects of WA on VEGF production in AGS cells induced by *H. pylori* infection, VEGF levels were measured in the culture supernatants using an ELISA. The results showed that pre-treatment as well as co-treatment with WA did not inhibit basal or *H. pylori*-induced production of VEGF in the cells (Fig. 3A and B). HIF-1 is a transcriptional factor that regulates a number of genes, including VEGF, involved in the hypoxic response (26). It is also known that *H. pylori* can induce the stabilization of HIF-1α to promote VEGF production (26-28). Therefore, the present study sought to determine the effects of WA on *H. pylori*-mediated HIF-1α stabilization using western blot analysis. As expected, *H. pylori* led to HIF-1α stabilization in gastric epithelial cells, which was not affected by pre-treatment of WA (Fig. 3C).

WA does not exhibit any anti-bacterial activity against H. pylori. To determine whether WA exerts any anti-bacterial activity against *H. pylori*, bacterial growth was evaluated by measuring their OD_{600} following incubation with WA. The results showed that the growth of *H. pylori* was not affected by WA, even at the high concentration of 1 μM. These results suggested that the anti-inflammatory activity of WA is not based on any bactericidal effect.

Discussion

IL-8 is a chemoattractant factor for neutrophil recruitment and a critical immune mediator for the pathogenesis of chronic gastritis caused by *H. pylori* infection. In addition, various studies have reported that high expression of IL-8 is correlated with poor prognosis of gastric cancers or gastrointestinal tumorigenesis, including angiogenesis (29-31). Therefore, IL-8 has been suggested as a therapeutic target in gastric cancer. The present study revealed that *in vitro* pre-treatment and co-treatment of WA effectively inhibits *H. pylori*-induced production of IL-8 in gastric epithelial cells, suggesting that WA may have preventive as well as therapeutic effects on *H. pylori*-mediated inflammation.

Various host factors, including phosphoinositide-3 kinase, heat shock protein 90, toll-like receptor 4, nicotinamide adenosine dinucleotide phosphate oxidase 1 and nucleotide-binding oligomerization domain 1 have been suggested as mechanisms for *H. pylori*-induced IL-8 production in gastric epithelial cells (32). NF-κB and MAPKs are known to be essential downstream molecules for the production of IL-8 induced by *H. pylori* (32). Previous studies by our and another group have also revealed that bacterial factors, including the type IV secretion system, are required for *H. pylori*-induced IL-8 production and activation of NF-κB and MAPKs (25,32). In the present study, pre-treatment with WA inhibited *H. pylori*-induced activation of NF-κB, but not MAPKs. It is known that WA inhibits NF-κB activation in a wide variety of cell types exposed to several stimuli, including LPS and TNF-α (33). By contrast, WA was shown to induce MAPK activation in breast cancer and leukemia cells (7,34,35), suggesting that WA may differentially regulate the activation of NF-κB and MAPKs in host cells. In the present study, although WA pre-treatment did not affect *H. pylori*-induced activation of MAPKs, the effect of WA on basal levels of MAPK activation in gastric cancer cells should not be ignored. It has been reported that WA induces apoptosis in various cancer types (33) and that MAPK-mediated signaling is involved in cellular apoptosis. In most experiments performed to evaluate the apoptotic effects of WA, a high concentration (>1 μM) of WA was used (7,34,35), whereas low concentrations (<500 nM) of WA were used in the present study. In fact, the MTT assay demonstrated that WA at concentrations >1 μM exerted cytotoxic effects on AGS cells (data not shown). Therefore, further experiments should be performed to clarify the effects of WA on gastric tumorigenesis.

VEGF is closely associated with poor prognosis of gastric cancer due to its characteristics of tumor invasion and lymph node metastasis (22,24). In addition, Wu *et al* (36) reported that NF-κB-mediated signaling is important for VEGF production induced by *H. pylori* in gastric epithelial cells. As the results indicated that WA inhibited *H. pylori*-induced NF-κB activation in AGS cells, the present study further assessed the effects of WA on VEGF production induced by *H. pylori*. It was

revealed that WA did not influence *H. pylori*-induced VEGF production in gastric epithelial cells. In a recent study by our group, an inhibitor assay revealed that NF-κB signaling is not essential for *H. pylori*-induced production of VEGF (25). Such VEGF production was reduced by digoxin (a HIF-1α inhibitor) and *N*-acetyl-L-cysteine, a scavenger of reactive oxygen species (ROS) (25). In a study by Zhu *et al* (37), the anti-oxidant compound pyrrolidine dithiocarbamate was used as an NF-κB inhibitor. These findings suggested that ROS and the HIF-1α axis are critical for *H. pylori*-induced VEGF production in gastric epithelial cells. In the present study, consistently with the results on VEGF production, HIF-1α stabilization by *H. pylori* was not affected by pre-treatment with WA. Therefore, it is expected that WA does not influence *H. pylori*-induced ROS production and any associated signaling.

Extracts from the leaf and root of *Withania somnifera* are known to have anti-bacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium* (38,39). Withanolides also inhibit the growth of *Proteus vulgaris* (40). These findings led to the speculation whether the inhibitory effects of WA on *H. pylori*-induced IL-8 production and NF-κB activation in gastric epithelial cells may be due to its bactericidal effects. However, in the present study, the growth of *H. pylori* was not affected by WA, suggesting that the anti-inflammatory activity of WA is due to direct inhibition of core signaling pathways, such as NF-κB, but not due to bactericidal effects.

In conclusion, the results of the present study showed that WA effectively inhibits *H. pylori*-induced IL-8 production and NF-κB activation in gastric epithelial cells. An *in vivo* study using a murine infection model and a clinical study are recommended to develop WA as a novel therapeutic agent or functional additive for the prevention on *H. pylori*-mediated inflammation or gastric diseases.

Acknowledgements

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