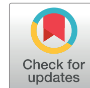

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Interruption of *Helicobacter pylori*-Induced NLRP3 Inflammasome Activation by Chalcone Derivatives

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Abstract

Helicobacter pylori causes chronic gastritis through *cag* pathogenicity island (*cag*PAI), vacuolating cytotoxin A (VacA), lipopolysaccharides (LPS), and flagellin as pathogen-related molecular patterns (PAMPs), which, in combination with the pattern recognition receptors (PRRs) of host cells promotes the expression and secretion of inflammation-causing cytokines and activates innate immune responses such as inflammasomes. To identify useful compounds against *H. pylori*-associated gastric disorders, the effect of chalcone derivatives to activate the nucleotide-binding oligomerization domain (NOD)-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome was examined in an *H. pylori*-infected human monocytic THP-1 cell line in this study. Among the five synthetic structurally-related chalcone derivatives examined, 2'-hydroxy-4',6'-dimethoxychalcone (8) and 2'-hydroxy-3,4,5-trimethoxychalcone (12) strongly blocked the NLRP3 inflammasome in *H. pylori*-infected THP-1 cells. At 10 μM, these compounds inhibited the production of active IL-1β, IL-18, and caspase-1, and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) oligomerization, but did not affect the expression levels of NLRP3, ASC, and pro-caspase-1. The interruption of NLRP3 inflammasome activation by these compounds was found to be mediated via the inhibition of the interleukin-1 receptor-associated kinase 4 (IRAK4)/IκBα/NF-κB signaling pathway. These compounds also inhibited caspase-4 production associated with non-canonical NLRP3 inflammasome activation. These results show for the first time that certain chalcones could interrupt the activation of the NLRP3 inflammasome in *H. pylori*-infected THP-1 cells. Therefore, these chalcones may be helpful in alleviating *H. pylori*-related inflammatory disorders including chronic gastritis.

Key Words: *Helicobacter pylori*, NLRP3, Inflammasome, Anti-inflammation, Chalcone derivatives

INTRODUCTION

Helicobacter pylori has infected more than 50% of the world's population and is classified as a class 1 carcinogen by the World Health Organization (WHO). *H. pylori* infection is known to cause chronic gastritis, peptic ulcers, gastric cancer, and mucosa-associated lymphoid tissue lymphoma (MALT) (Çalişkan *et al*., 2015). The *cag* pathogenicity island (*cag*PAI) and vacuolating cytotoxin A (VacA) of *H. pylori* cause host inflammatory reactions, which are associated with an increase in gastric ulcers and gastric cancer (Miernyk *et al*., 2011; Semper *et al*., 2014). Cytotoxin-associated gene A (CagA) and VacA migrate to the epithelial cells of the host by a type IV secretion system (T4SS) and a type V secretion system (T5SS) encoded by *cag*PAI (Alzahrani *et al*., 2014; Kumar and Dhiman, 2018). In addition, *H. pylori* infection with pathogen-associated molecular patterns (PAMPs), including lipopolysaccharides (LPS) and flagellin, activates the innate immune response. When PAMPs are combined with pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) to activate receptors, they promote the expression and secretion of pro-inflammatory cytokines by immune cells. In previous studies, the upregulation of IL-1β and IL-18 was demonstrated in human immune cells and the gastric tissue of animal models, further indicting that *H. pylori* infection activates the inflammasome (Semper *et al*., 2014; Li *et al*., 2015; Kameoka *et al*., 2016; Pérez-Figueroa *et al*., 2016).

Inflammasomes, as components of the innate immune responses, are multimeric protein complexes formed in response to many inflammatory stimuli. Among the inflammasome complexes reported so far, NOD-like receptor family, pyrin domain-containing 3 (NLRP3) is involved in several chronic inflammatory diseases such as arthritis and colitis,

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and the increased expression of NLRP3 was reported in gastric cancer (Guo *et al*., 2018; Moossavi *et al*., 2018; Perera *et al*., 2018). When immune cells, especially macrophages and dendritic cells, are infected with *H. pylori*, the elevated IL-1β production by activation of the NLRP3 inflammasome initiates an inflammatory reaction such as neutrophil infiltration and decreases gastric acid secretion, helping the bacteria colonize and survive long-term in the gastric tissue (Israel and Peek, 2018; Pachathundikandi *et al*., 2019). In contrast, the inhibition of caspase-1 involved in the maturation of IL-1β and IL-18 blocks the NLRP3 inflammasome pathway, reducing gastric inflammation (Hitzler *et al*., 2012). Thus, finding new inhibitors that can regulate NLRP3 components such as IL-1β, IL-18, and caspase-1, and signaling molecules related to the activation of the NLRP3 inflammasome pathway may help control inflammation and the immune response in gastric inflammatory diseases induced by *H. pylori* infection.

Chalcones are widely distributed in vegetables, fruits, teas, and other plants, belong to the family of flavonoids, and act as intermediates in the biosynthesis of flavonoids as a precursor to flavones (Jandial *et al*., 2014). Chalcones have been reported to possess various pharmacological activity including analgesic, antioxidant, anti-inflammatory, anti-cancer (De Campos-Buzzi *et al*., 2007), antibacterial, antifungal, and antiviral effects (Nowakowska, 2007). The chalcone-based compound, metochalcone (Sahu *et al*., 2012), is approved as a choleretic drug and sofalcone (Higuchi *et al*., 2010), having a chalcone structure, has been used as an ulcer and mucosal protection drug. Recently, the natural chalcone, isoliquiritigenin, was demonstrated to affect the inflammasome pathway (Honda *et al*., 2014; Zeng *et al*., 2017; Nakamura *et al*., 2018). This is the only report describing the inhibitory action of a chalcone derivative on inflammasome formation. It is not known whether some other chalcones may possess a similar activity and the detailed action mechanisms for the biological/pharmacological activities of chalcone derivatives have not been elucidated. Furthermore, there has been no report on the effect of chalcones in *H. pylori* infection and gastritis. Therefore, the present study investigated the inhibitory mechanisms of selected chalcone derivatives against the *H. pylori*-induced activation of the NLRP3 inflammasome and the inflammatory network and suggests a potential use in *H. pylori*-induced gastric disorders.

MATERIALS AND METHODS

Preparation of the chalcone derivatives

All tested chalcone derivatives except isoliquiritigenin were prepared following known methods as previously described (Dao *et al*., 2003; Dao, 2004).

Chemicals

Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from Hyclone Laboratories (South Logan, UT, USA). Fetal bovine serum (FBS) was obtained from MP Biomedicals (Santa Ana, CA, USA). Opti-MEM, glutamine and penicillin/streptomycin were the products of Gibco (Grand island, NY, USA). Isoliquiritigenin (1), phorbol 12-myristate 13-acetate (PMA), phenylmethylsulfonyl fluoride (PMSF), polyvinylidene fluoride (PVDF), PF 06650833 (IRAK4 inhibitor), Bay 11-7082 (NF-κB inhibitor) and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). Suberic acid bis (3-sulfo-N-hydroxysuccinimide ester) sodium salt (BS^3) was bought from BioVision (Milpitas, CA, USA). Lactate dehydrogenase (LDH) cytotoxicity WST assay kit and Z-YVAD(Ome)-FMK (caspase-1 inhibitor) were obtained from Enzo Life Sciences (Farmingdale, NY, USA). Pro-prep solution was obtained from iNtRON Biotechnology (Seongnam, Korea). All primary antibodies relating NLRP3 inflammasome and signaling pathway and secondary antibodies were bought form Cell Signaling Technology (Minneapolis, MN, USA). Primary anti-β-actin antibody was obtained from Bethyl Laboratories (Montgomery, TX, USA). IL-1β ELISA kit was purchased from BD Biosciences (San Jose, CA, USA). IL-18 ELISA kit was bought from R&D Systems (Minneapolis, MN, USA).

H. pylori infection

Human monocytic cell line THP-1 (ATCC, Manassas, VA, USA) was cultured in RPMI 1640 medium containing 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin in a 5% CO_2, 37°C humidified incubator. To induce differentiation, THP-1 cells were treated with 0.5 μM PMA for 3 h. After overnight incubation, cells were seeded in 96-well plates (1×10^5 cells/well), 24-well plates (1×10^6 cells/well), or 6-well plates (3×10^6 cells/well) for further experiments.

H. pylori strain ATCC 43504 (ATCC, Manassas, VA, USA) was cultured on Blood Agar plate (Synergy Innovation, SeongNam, Korea) at 37°C in a 10% CO_2 incubator under microaerophilic conditions. After 2-3 days, liquid cultures were maintained in Brucella broth (Sigma-Aldrich) containing 10% horse serum (Gibco). For infection with *H. pylori* in THP-1 cells, the bacteria was centrifuged at 12,000 rpm for 5 min. After re-suspended in Opti-MEM, they were added to the cells at a multiplicity of infection (MOI=25) for 6 h.

Cytotoxicity was determined by LDH cytotoxicity WST assay according to the manufacturer's instructions (Enzo Life Sciences). All experiments were performed at the concentrations where chalcone derivatives were non-toxic to the cells, and all experiments were repeated three times.

Measurement of minimum inhibitory concentration (MIC) of chalcone derivatives

MIC was measured in a 96-well plate through the microdilution broth method. *H. pylori* strain ATCC 43504 was incubated for 2-3 days at 37°C and 10% CO_2 on a Blood Agar plate. After liquid culture in Brucella broth containing 10% horse serum, it was seeded at 5×10^5 CFU/well. The chalcone derivatives were treated by 2-fold dilution from 200 μM to 12.5 μM. Amoxicillin (Sigma-Aldrich) was used as a reference compound at 2 μg/mL. Then, it was incubated for 72 h under the conditions of 10% CO_2 and 37°C, and optical density (OD) was measured at 550 nm. After subtracting the OD value of blank (only Brucella broth) from each well, the MIC was determined using the following equation: (OD_{550} of wells that contained the compound/OD_{550} of the compound-free well)×100.

Measurements of NLRP3 inflammasome activation

THP-1 cells were differentiated with 0.5 μM PMA for 3 h and then infected with *H. pylori* at 25 MOI for 6 h after pretreatment of each chalcone derivative (10 μM) for 30 min. IL-1β and IL-18 ELISA in cell culture supernatants were performed according to the manufacturer's instructions. Western

Blotting was performed to measure the expression levels of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), NLRP3, pro-IL-1β and pro-caspase-1 proteins in cell lysates. According to the previously established procedure (Lim *et al*., 2018), mature forms of caspase-1 and IL-1β in the cell culture supernatant were concentrated using the methanol/chloroform method, and ASC oligomerization experiment was performed using suberic acid bis (3-sulfo-N-hydroxysuccinimide ester) sodium salt (BS^3) in cell lysates. Protein concentrations were measured using the Bradford solution (Bio-rad Lab., Hercules, CA, USA). The same protein amounts were loaded and then were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, bands were transferred to a PVDF membrane using Pierce G2 Fast blotter (Thermo Fisher Scientific, Rockford, IL, USA). The blots were blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween 20 (TBST) and incubated with primary antibody (1:1000) for overnight at 4°C. The membrane was incubated with secondary antibody in 5% skim milk (1:5000) for 1 h and washed out with TBST for 5 times every 5 min. Protein bands were detected by enhanced chemiluminescence (ECL) system (West Femto Luminol/Enhancer solution, Thermo Fisher Scientific) and quantified using FUSION FX chemiluminescence (VILBER, *Marne-la-Vallee cedex* 3, France). The expression level of each protein was normalized to β-actin. All compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted to be 0.1% (v/v) at a final concentration.

Cellular mechanism study

PMA-differentiated THP-1 cells were infected with *H. pylori* at 25 MOI for 15, 30, and 60 min after pretreatment of each chalcone derivative at 10 μM for 30 min. To examine the interleukin-1 receptor-associated kinase 4 (IRAK4) phosphorylation, mitogen-activated protein kinase (MAPK) activation, and the degradation and phosphorylation of IκBα in total cell lysates, cellular proteins were extracted using Pro-prep solution containing 1 mM PMSF, 1 mM sodium orthovanadate and 1 mM sodium fluoride (NaF). Proteins were quantified with Bradford solution and then were separated in SDS-PAGE. The expression of transcription factors such as the activation levels of NF-κB and c-Jun were identified in nuclear fraction using Western blotting. Nuclear proteins were extracted and quantified via bicinchoninic acid assay (BCA assay, Thermo Fisher Scientific). Western blotting was performed as same as the described above.

Statistical analysis

All experimental data were presented as arithmetic mean ± SD. Statistical significance was determined using the one way

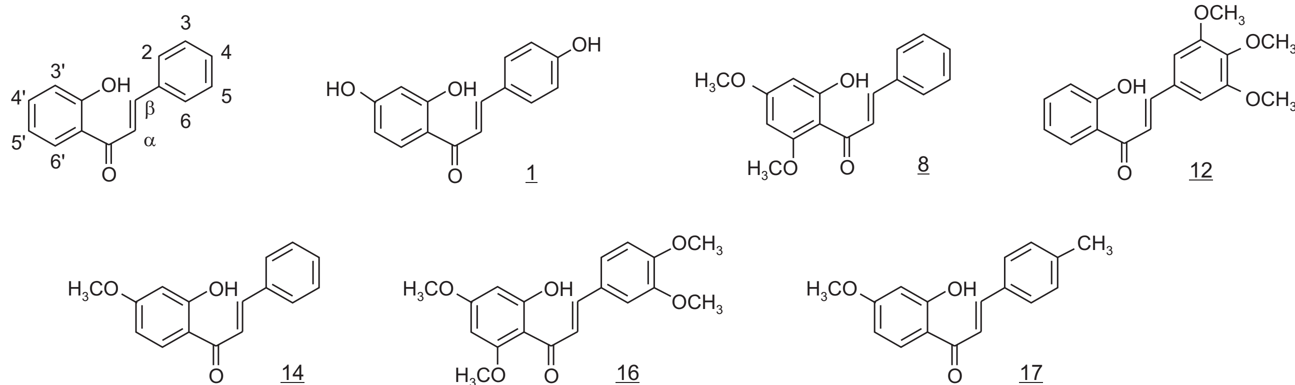

Fig. 1. Chemical structures of 5 chalcone derivatives tested in this study.

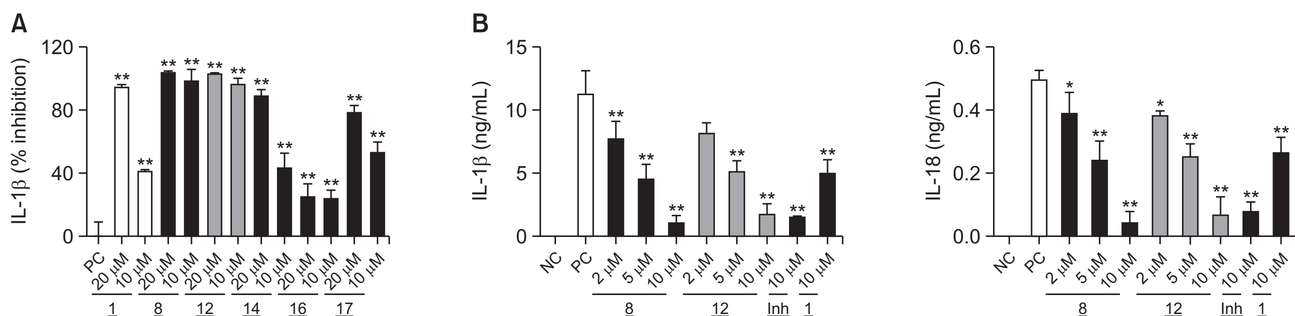

Fig. 2. Inhibition of IL-1β and IL-18 production of the chalcone derivatives in *H. pylori*-treated THP-1 cells. Chalcone derivatives were pretreated for 30 min prior to treating with *H. pylori* (MOI=25) in THP-1 cells. After 6 h, the concentrations of active IL-1β and/or IL-18 protein in cell culture supernatant were measured using ELISA (n=3). (A) Effect of 5 chalcone derivatives (10 and 20 μM) on active IL-1β production. (B) Effect of the compound 8 and 12 (2, 5, and 10 μM) on active IL-1β and IL-18 production in *H. pylori*-treated THP-1 cells. NC: negative control (*H. pylori*-untreated cells), PC: positive control (*H. pylori*-treated cells), Inh: Z-YVAD(OMe)-FMK (caspase-1 inhibitor), 1: Isoliquiritigenin, 8 to 17: compound 8 to 17. *p<0.05, **p<0.01, significantly different from PC.

ANOVA following Dunnett's test (IBM SPSS Statistics version 24, IBM, Armonk, NY, USA).

RESULTS

Effects of chalcone derivatives on active IL-1β production in monosodium urate (MSU)-induced THP-1 cells

To investigate the effect of synthetic chalcones on the activation of the NLRP3 inflammasome, the inhibitory effect of 15 synthetic chalcone derivatives including isoliquiritigenin (1) on IL-1β production in MSU crystal-induced THP-1 cells was examined, which is one of the NLRP3 inflammasome activation models used in our previous report (Lim *et al*., 2018). At 10 μM, most of the chalcones decreased IL-1β production (> 19.6%), an indicator of the activation of the NLRP3 inflammasome (Supplementary Fig. 1).

Effects of chalcone derivatives on active IL-1β and IL-18 production in *H. pylori*-treated THP-1 cells

Based on the above findings, five synthetic chalcone derivatives were selected (Fig. 1) and the effects on mature IL-1β production were examined in *H. pylori*-treated THP-1 cells since fully differentiated THP-1 cells produce mature IL-1β by activation of the NLRP3 inflammasome by *H. pylori* infection (Fig. 2A). Four chalcones except compound 16 strongly inhibited IL-1β production at 10–20 μM. Among them, two chalcones (8 and 12) with the most potent inhibitory activity (>90% inhibition at 10 μM) were selected for further study. They significantly inhibited the secretion of active IL-1β and IL-18 in a concentration-dependent manner at 2, 5, and 10 μM (Fig. 2B). The secretion of mature IL-1β into the supernatant was inhibited by 88.2% (8) and 81.8% (12) at 10 μM and the secretion of active IL-18 was reduced by 88.7% and 84.2% at 10 μM, respectively. When the cells were treated with 10 μM isoliquiritigenin (1), previously reported as an NLRP3 inflammasome inhibitor, and a caspase-1 inhibitor (Inh; Z-YVAD(Ome)-FMK) under the same experimental conditions, the concentration of cleaved IL-1β was reduced by 50.0% and 83.9% and IL-18 was inhibited by 55.4% and 82.0%, respectively. These results suggest that two chalcones (8 and 12) found in this study exerted inhibitory effects against NLRP3 activation and were more potent inhibitors of the NLRP3 inflammasome than isoliquiritigenin.

Effects of chalcone derivatives (8 and 12) on NLRP3 inflammasome activation

After the THP-1 cells were differentiated with PMA, the cells were pretreated with compounds 8 and 12 at 10 μM for 30 min and infected with *H. pylori* at 25 MOI for 6 h. To confirm the NLRP3 inflammasome activation and inhibitory action of the chalcones, the protein expression of the components of the NLRP3 inflammasome and ASC oligomerization was analyzed in the supernatants and cell lysates by Western blots. As expected, *H. pylori* infection increased the protein expression levels of NLRP3 inflammasome-related molecules. Under these conditions, compounds 8 and 12 did not affect the protein expression levels of NLRP3 inflammasome-related molecules, pro-IL-1β, pro-caspase-1, and NLRP3 in the cell lysates. In contrast, treatment with compounds 8 and 12 affected the levels of cleaved IL-1β (17 kDa) and active caspase-1 protein (20 kDa) secreted into the cellular supernatant (Fig. 3). At 10 μM, compound 8 decreased the concentrations of cleaved IL-1β and active caspase-1 by 94.5% and 69.7%, respectively, and 12 decreased the expression by 56.8% and 59%, respectively. In addition, neither compound affected ASC protein expression, but ASC oligomerization including dimers and oligomers, direct evidence of activation of the NLRP3

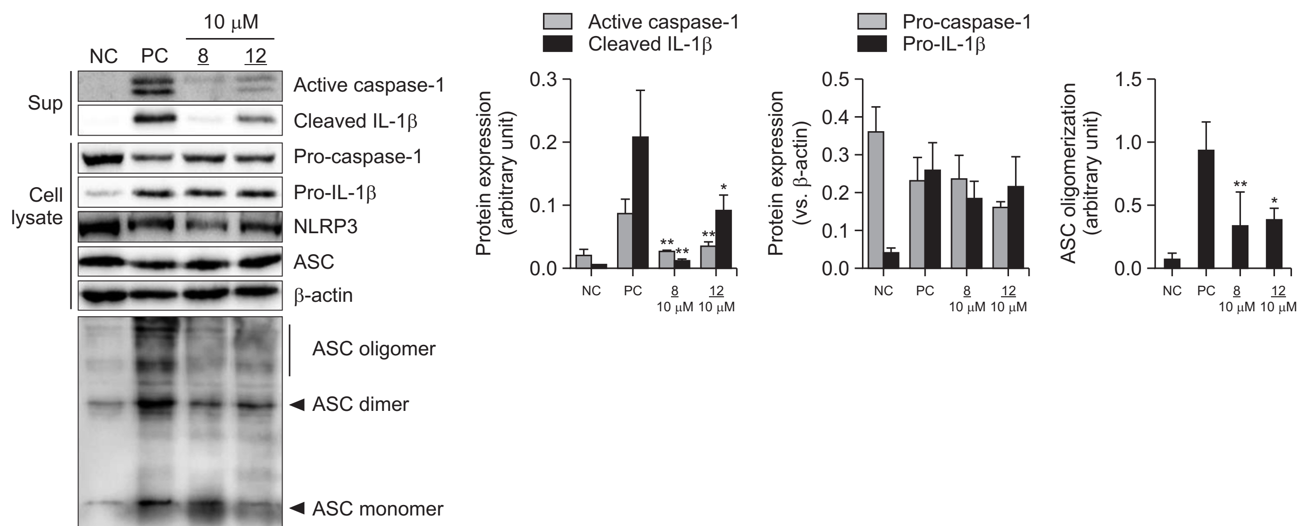

Fig. 3. Effect on the components of NLRP3 inflammasome and ASC oligomerization of 8 and 12 in *H. pylori*-treated THP-1 cells. For activation of NLRP3 inflammasome, PMA-differentiated THP-1 cells were infected with *H. pylori* at 25 MOI for 6 h after pretreatment of each chalcone derivative at 10 μM for 30 min. The ASC oligomerization level was measured through Western blotting as described in the materials and methods. The expression of pro-IL-1β and pro-caspase-1, NLRP3, and ASC were examined in total cell lysates and cleaved IL-1β (17 KDa) and active caspase-1 (20 KDa) were analyzed in the supernatant using Western blotting. Each blot is a representative among three separate experiments. NC: negative control (*H. pylori*-untreated cells), PC: positive control (*H. pylori*-treated cells), **p*<0.05, ***p*<0.01, significantly different from PC.

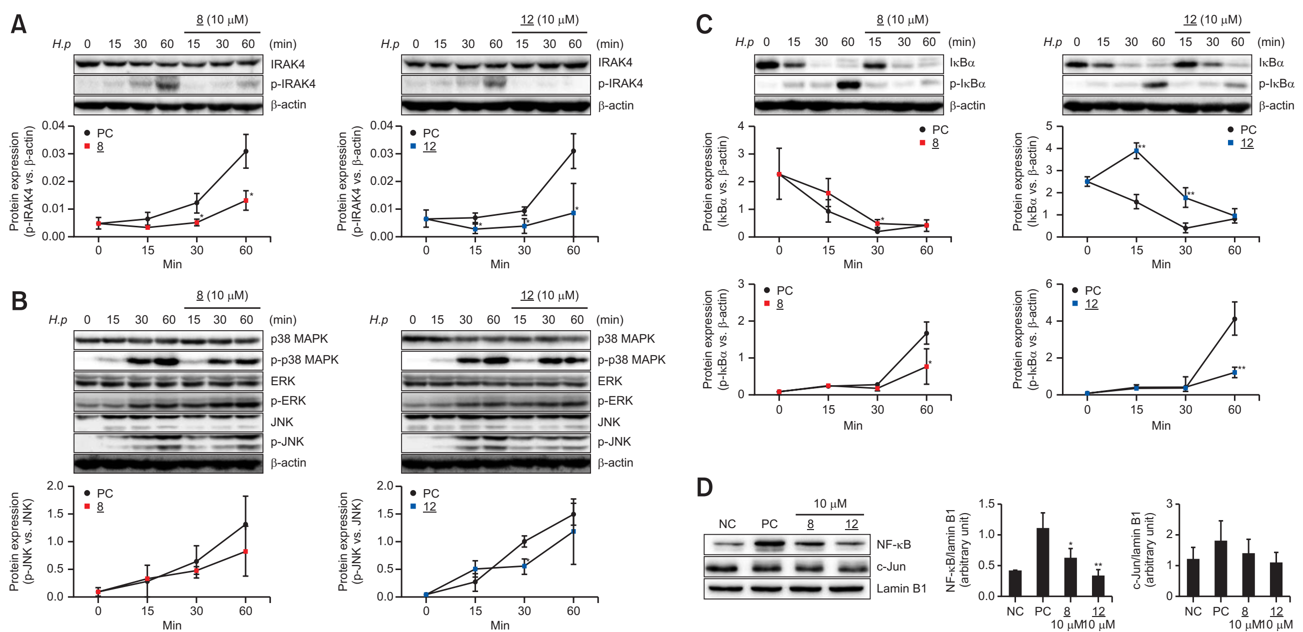

Fig. 4. Effect of 8 and 12 on cell signaling pathway in *H. pylori*-treated THP-1 cells. Chalcone derivatives were pretreated for 30 min in THP-1 cells and then treated with *H. pylori* (MOI=25) for 0, 15, 30 and 60 min. Levels of phosphorylation of IRAK4 and MAPKs (p38 MAPK, ERK, and JNK) in cell lysate were examined using Western blotting and the blots were normalized to total protein level. Degradation and phosphorylation of IκBα were normalized to β-actin protein level. After *H. pylori* treatment in the cell for 30 min, the amounts of NF-κB p65 and c-jun translocated to the nucleus were examined by Western blotting. Nuclear proteins were normalized to Lamin B1. (A) Effects of 8 and 12 (10 μM) on total expression of IRAK4 and the phosphorylation of IRAK4. (B) Effects of 8 and 12 (10 μM) on the phosphorylation of MAPKs. (C) Effects of 8 and 12 (10 μM) on the degradation and phosphorylation of IκBα. (D) Inhibition of translocation to nucleus of NF-κB p65 and c-jun by 8 and 12. Each blot is a representative among three separate experiments. NC: negative control (*H. pylori*-untreated cells), PC: positive control (*H. pylori*-treated cells), *p<0.05, **p<0.01, significantly different from PC.

inflammasome, was significantly reduced by treatment with compound 8 by 64% and compound 12 by 59.1%.

The inhibitory action of the chalcones against *H. pylori*-induced NLRP3 inflammasome activation may be due to their bactericidal action. To investigate this possibility, a MIC experiment was conducted. When 8 and 12 at 12.5–200 μM were treated with *H. pylori* for 72 h, there was no decrease in bacterial survival (Supplementary Table 1). Thus, the strong inhibition of the *H. pylori*-induced NLRP3 inflammasome activation by these chalcones was not due to the intrinsic toxicity of the tested chalcones against *H. pylori*.

All these results indicate that compounds 8 and 12 inhibited the activation of the NLRP3 inflammasome by blocking the cleavage of the IL-1β and IL-18 protein proforms into active forms and ASC oligomerization without affecting the protein expression levels of the components of the NLRP3 inflammasome including pro-IL-1β, pro-caspase-1, NLRP3, and ASC.

Elucidation of the cellular mechanisms of the chalcone derivatives in the inhibition of *H. pylori*-induced NLRP3 inflammasome activation

To examine the signaling pathway involved in the inhibition of the activation of the NLRP3 inflammasome by compounds 8 and 12, the effects on IRAK4, MAPK, IκBα, and NF-κB were analyzed using Western blots. These molecules were previously reported to be closely associated with NLRP3 inflammasome activation (Man and Kanneganti, 2015; Tóth *et al*., 2017; An *et al*., 2019). THP-1 cells differentiated by PMA were treated with *H. pylori* (MOI=25) for 15, 30, and 60 min. As shown in Fig. 4A, IRAK4 phosphorylation was increased over time by treatment with *H. pylori*. When the cells were treated with 8 and 12 (10 μM) and *H. pylori*, IRAK4 phosphorylation was inhibited by 57.2% and 72.9%, respectively, at 60 min. When the inhibitory activity of the chalcones at 10 μM on the MAPK pathway including p38 MAPK, extracellular-signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) was examined, neither 8 nor 12 affected the phosphorylation of p38 MAPK and ERK. However, compound 12 significantly reduced JNK phosphorylation by 45.4% (Fig. 4B). Next, to identify the effect of these two chalcones on the NF-κB signaling pathway, the level of degradation and phosphorylation of the IκBα protein was initially examined since it was previously shown that the total IκBα protein was degraded and the level of p-IκBα was increased when NF-κB was activated (Wang *et al*., 2012). Our data also demonstrated that the total IκBα expression was decreased by infection with *H. pylori*, whereas the phospho-form of IκBα was increased (Fig. 4C). Under these conditions, when the cells were treated with each chalcone (10 μM) and *H. pylori*, the degradation of IκBα was slower than that of the positive control (PC). In particular, 12 showed stronger activity than 8 in blocking IκBα degradation. In addition, the phosphorylation of IκBα was strongly inhibited by treatment with compounds 8 (54.1%) and 12 (70.3%) for 60 min. Next, to confirm if the translocation of the transcription factors into the nucleus was inhibited, the concentrations of NF-κB p65 and c-Jun in the nuclear fraction were analyzed by Western blots. THP-1 cells were treated with 8 and 12 for 30 min and infected with *H. pylori* for 30 min. As expected,

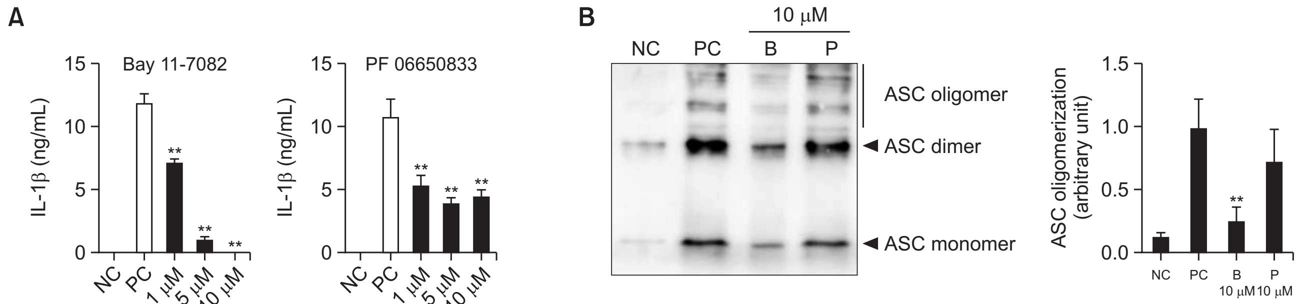

Fig. 5. Inhibitory effect of Bay 11-7082 and PF 06650833 in *H. pylori*-treated THP-1 cells. PMA-differentiated THP-1 cells pretreated for 30 min with Bay 11-7082 and PF 06650833 (1, 5, and 10 μM) were infected with *H. pylori* (MOI=25) for 6 h. The active IL-1β was measured using ELISA in cell culture supernatant. ASC oligomerization was measured by Western blotting in cell lysate. (A) Inhibition of Bay 11-7082 and PF 06650833 on active IL-1β production. (B) Effect of Bay 11-7082 and PF 06650833 on ASC oligomerization. B: Bay 11-7082 (NF-κB inhibitor), P: PF 06650833 (IRAK4 inhibitor). NC: negative control (*H. pylori*-untreated cells), PC: positive control (*H. pylori*-treated cells). $^{**}p<0.01$, significantly different from PC.

the translocation of NF-κB p65 into the nucleus was reduced by 43.4% by treatment with compound 8 and 69.9% by treatment with compound 12 at 10 μM (Fig. 4D). However, the activation of c-Jun, a component of activator protein 1 (AP-1), was not changed under the same experimental conditions. All these results suggest that suppression of the activation of the NLRP3 inflammasome by the two chalcones was mediated via the IRAK4/IκBα/NF-κB signaling pathway.

To confirm if the signaling molecules identified in this study were actually associated with activation of the NLRP3 inflammasome, the effects of an NF-κB inhibitor and an IRAK4 inhibitor on the levels of active IL-1β production were measured (Fig. 5). As expected, both of these inhibitors strongly reduced active IL-1β production. Bay 11-7082 (NF-κB inhibitor) at 10 μM and PF 06650833 (IRAK4 inhibitor) at 10 μM decreased the production by 97.9%and 56.9%, respectively. Furthermore, Bay 11-7082 and PF 06650833 also inhibited ASC oligomerization by 75.2% and 26.6%, respectively. The inhibition by the NF-κB inhibitor was more potent than that of the IRAK4 inhibitor. Thus, these results indicate that the IRAK4 and NF-κB pathway was closely related to the activation of the NLRP3 inflammasome provoked by *H. pylori* infection in the THP-1 cells.

Besides the canonical NLRP3 inflammasome pathway described above, several molecules related to the non-canonical pathway have been recently reported (Casson *et al*., 2015; Viganò *et al*., 2015). Accordingly, the expression level of caspase-4, a representative marker of the non-canonical NLRP3 pathway, was measured in the cellular supernatants. Compounds 8 and 12 reduced the secretion of cleaved caspase-4 into the cell supernatants by 78.1% and 29.3%, respectively, whereas no significant difference in the expression of pro-caspase-4 in the cell lysates was found (Fig. 6). All these results demonstrate that chalcones 8 and 12 inhibited the canonical as well as the non-canonical NLRP3 inflammasome pathway.

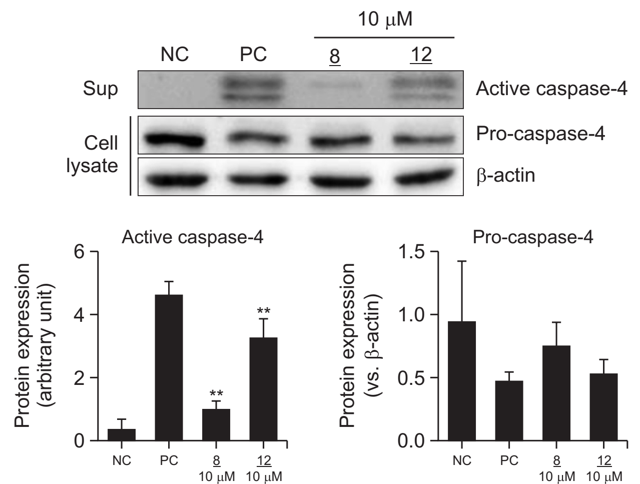

Fig. 6. Effect of 8 and 12 on alternative NLRP3 inflammasome in *H. pylori*-induced THP-1 cells. THP-1 cells were pretreated with 8 and 12 (10 μM) for 30 min and then infected with *H. pylori* for 6 h. Pro-caspase-4 in total cell lysate and active caspase-4 in cell supernatant were detected in cell supernatant through Western blotting. Pro-caspase-4 was normalized to β-actin protein level. NC: negative control (*H. pylori*-untreated cells), PC: positive control (*H. pylori*-treated cells). $^{**}p<0.01$, significantly different from PC.

DISCUSSION

This study showed for the first time that certain chalcone derivatives could interrupt *H. pylori*-induced NLRP3 inflammasome activation and that this inhibition was possibly mediated by interaction with the IRAK4/IκBα/NF-κB pathway.

When *H. pylori* infects the stomach, surface TLRs of the gastric epithelial and immune cells of the host including macrophages recognize various gastric pathogens of *H. pylori* such as CagA, VagA, LPS, and flagellin as various PAMPs (Castaño-Rodríguez *et al*., 2014). Previous studies have shown that TLR2 and TLR4 were critical signaling receptors in the activation of *H. pylori*-induced host immune responses (Obonyo *et al*., 2007). *H. pylori* PAMPs form a complex with TLRs, and these complexes induce the myeloid differentiation primary response 88 (MyD88)-dependent signaling pathway and activate the production of cytokines such as IL-1β and TNF-α (Takeda and Akira, 2015). MyD88 recruited via TLR

activates IRAK4, which forms a complex. This leads to the activation of IκB kinase (IKK) and the phosphorylation and degradation of IκBα protein. In a subsequent reaction, NF-κB migrates to the nucleus and activates transcription (Li, 2008). The essential role of IRAK4 in the inflammatory process was revealed through a knockout mouse model study (Koziczak-Holbro *et al*., 2007; Flannery and Bowie, 2010). $IRAK4^{-/-}$ mice showed complete resistance to LPS-induced septic shock and the cytokine response was inhibited. It was also confirmed that the IL-1-induced NF-κB, JNK, and p38 MAPK activation was also inhibited in cells derived from the same knockout mice. Thus, IRAK4 was crucial in provoking *H. pylori*-induced inflammatory responses and the activation of NF-κB. In the present study, when *H. pylori*-infected THP-1 cells were treated with compounds 8 and 12, the phosphorylation and degradation of IκBα and IRAK4 in the NF-κB signaling pathway were significantly reduced (Fig. 4A, 4C). The involvement of NF-κB in the activation of the NLRP3 inflammasome by *H. pylori* infection was also confirmed in the present study (Fig. 5) and both 8 and 12 also inhibited the translocation of NF-κB p65 into the nucleus (Fig. 4D). All these findings indicate that IRAK4 and NF-κB signaling was essential for *H. pylori*-induced inflammasome activation and suggested that the mediation of the IRAK4/IκBα/NF-κB pathway by chalcones 8 and 12 could lead to reductions in the NLRP3 inflammasome activation by *H. pylori* infection.

Chalcones 8 and 12 had little effect on the expression levels of pro-IL-1β and pro-caspase-1 proteins, which are components of the NLRP3 inflammasome, but showed strong inhibitory activity against ASC oligomerization, a direct indicator of NLRP3 inflammasome activation (Fig. 3). This indicated that both chalcones were involved in the canonical NLRP3 inflammasome pathway through the inhibition of caspase-1 activation. It also confirmed that the chalcones interrupted the non-canonical NLRP3 inflammasome pathway through the downregulation of caspase-4 (Fig. 6). Human caspase-4/5 is known to mediate the activation of non-canonical inflammasome pathways (Viganò *et al*., 2015). In a recent study, caspase-4-deficient THP-1 cells were found to be resistant to LPS stimulation (Schmid-Burgk *et al*., 2015) and caspase-4 mediated pyroptosis in human monocytes (Shi *et al*., 2014). This pro-inflammatory caspase (caspase-1/4/5) is structurally similar to caspase-8/9 which initiates apoptosis. Thus, it is clear that 8 and 12 are involved in the canonical/non-canonical pathways, reducing the activation of the NLPR3 inflammasome.

Chalcones are considered a type of flavonoid. Chemically, they have open B-ring structures. Although detailed structure-activity relationships could not be drawn from the present study mainly because of the small numbers of chalcones tested, this investigation clearly demonstrated that certain chalcones could inhibit *H. pylori*-induced NLRP3 inflammasome activation. The inhibitory activity of flavonoids and chalcones against inflammatory molecules have been proved as transcription regulators in many inflammatory conditions. Flavonoids such as apigenin, quercetin, or natural products containing flavonoids as main ingredients showed anti-inflammatory activity in the treatment of gastric inflammation in several animal models (González-Segovia *et al*., 2008; Kuo *et al*., 2014; Zhang *et al*., 2015; Kim *et al*., 2016). There have been a few reports concerning the anti-inflammatory (Lai *et al*., 2010) and anti-tumor (Lin and Shih, 2014) effects of chalcones in human gastric cells. The only chalcone reported as the inhibitor of the NLRP3 inflammasome is isoliquiritigenin (Honda *et al*., 2014; Liu *et al*., 2017; Zeng *et al*., 2017; Nakamura *et al*., 2018). In this regard, elucidating the inhibitory effect of certain chalcones on the NLRP3 inflammasome in the present investigation is important, and identifying the signaling target molecules related to the NLRP3 inflammasome pathway could represent a new action point of the anti-inflammatory chalcones.

As stated above, *H. pylori* contributes to several gastric disorders and NLRP3 inflammasome activation induced by *H. pylori* infection in the gut may deteriorate the inflammatory response. Certain compounds controlling these processes, like chalcones in this study, may show beneficial effects against *H. pylori* infection in combination with or without antibiotic therapy. To confirm this notion, a well-designed *in vivo* study is needed.

In conclusion, the present study suggested that certain chalcone derivatives inhibited the activation of the NLRP3 inflammasome in *H. pylori*-infected THP-1 cells. This inhibition was mediated by interrupting the IRAK4/IκBα/NF-κB signaling pathway. Therefore, blocking inflammasome activation using certain chalcone derivatives as a first defense in *H. pylori*-infected gastric cells can help slow the progression of gastric inflammation into chronic gastritis and further gastric cell transformation into gastric cancer.

CONFLICT OF INTEREST

There are no conflicts of interest.

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