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Original Research Article

Anti-Inflammatory Activity of Acacia Honey through Inhibition of NF-κB and MAPK/ATF2 Signaling Pathway in LPS-Stimulated RAW264.7 Cells

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Abstract - Honey used as conventional medicine has various pharmacological properties. In the honey and anti-inflammatory effect, Gelam honey and Manuka honey has been reported to exert anti-inflammatory activity. However, the anti-inflammatory effect and potential mechanisms of acacia honey (AH) are not well understood. In this study, we investigated anti-inflammatory activity and mechanism of action of AH in LPS-stimulated RAW264.7 cells. AH attenuated NO production through inhibition of iNOS expression in LPS-stimulated RAW264.7 cells. AH also decreased the expressions of IL-1β, IL-6 and TNF-α as pro-inflammatory cytokines, and MCP-1 expression as a pro-inflammatory chemokine. In the elucidation of the molecular mechanisms, AH decreased LPS-mediated IκB-α degradation and subsequent nuclear accumulation of p65, which resulted in the inhibition of NF-κB activation in RAW264.7 cells. AH dose-dependently suppressed LPS-mediated phosphorylation of ERK1/2 and p38 in RAW264.7 cells. In addition, AH significantly inhibited ATF2 phosphorylation and nuclear accumulation of ATF2 in LPS-stimulated RAW264.7 cells. These results suggest that AH has an anti-inflammatory effect, inhibiting the production of pro-inflammatory mediators such as NO, iNOS, TNF-α, IL-6, IL-1β and MCP-1 via interruption of the NF-κB and MAPK/ATF2 signaling pathways.

Key words – Acacia honey, Anti-inflammation, Honey, Pro-inflammatory mediators

Introduction

Inflammation is known to be a defensive response against harmful pathogens, but chronic inflammation induced by uncontrolled inflammatory response has been reported to be associated with the pathogenesis of various human inflammatory diseases such as gingivitis, cardiovascular disease, cancer, arthritis, asthma allergies, alzheimer's disease, ulcerative colitis and inflammatory bowel disease (Medzhitov, 2008; Nho *et al*., 2018; Park *et al*., 2018). Although various anti-inflammatory drugs such as aspirin, celecoxib, ibuprofen, naproxen and diclofenac have been developed, the discovery of the less toxic and effective anti-inflammatory agents are still required because of the side effect of the current anti-inflammatory drugs (Gasparrini *et al*., 2018).

Honey has been used as conventional medicine for wound healing and anti-bacteria from ancient time and has been known to have various pharmacological activities such as anti-cancer (Afrin *et al*., 2018a; Afrin *et al*., 2018b), immune response (Al-Waili and Haq, 2004) and cardioprotective activity (Alvarez-Suarez *et al*., 2013). In addition, honey has been reported as an important resource for the development of anti-inflammatory drugs (Khan *et al*., 2018). In the study for anti-inflammatory activity of honey, Malaysian honey has been reported to inhibit NO production in LPS or IFN-γ-stimulated RAW264.7 cells (Kassim *et al*., 2010). Gelam honey inhibited ovalbumin-induced airway inflammation in a mice model of allergic asthma (Shamshuddin and Mohd Zohdi, 2018). In addition, Manuka honey blocked the expression of the pro-inflammatory mediators through inhibiting Keap1/Nrf2 and TLR4/NF-κB signaling pathways (Gasparrini *et al*., 2018). However, the anti-inflammatory activity and the

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mechanism of action of acacia honey have not been elucidated.

In this study, we aimed to investigate anti-inflammatory activity of acacia honey in LPS-stimulated RAW264.7 cells, and to elucidate the potential mechanism.

Materials and Methods

Chemical reagents

Dulbecco's Modified Eagle medium (DMEM)/F-12 1:1 Modified medium (DMEM/F-12) for cell culture was purchased from Lonza (Walkersville, MD, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for cell viability and lipopolysaccharide (LPS) for the induction of inflammatory response ware purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies against IκB-α, p65, phospho-ERK1/2, ERK1/2, phospho-p38, p38, p-ATF2, ATF2, β-actin and TBP were purchased from Cell Signaling (Bervely, MA, USA).

Sample preparation

Honey samples according to the honey plants were kindly provided from Chuleui Jung as a professor at the Department of Plant Medicine in Andong National University, Korea. One hundred gram of each honey was extracted with 200 ㎖ of ethylacetate three times. Ethylacetate extracts from each honey were concentrated to a vacuum evaporator and then freeze-dried. The ethylaeetate extracts from each honey were kept in a refrigerator until use.

Cell culture and treatment

RAW264.7 cells as a murine macrophage cell line were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained at 37℃ under a humidified atmosphere of 5% CO_2 using DMEM/F-12 media containing 10% fetal bovine serum (FBS), 100 U/㎖ penicillin and 100 ㎍/㎖ streptomycin. The ethylaeetate extracts from each honey were dissolved in dimethyl sulfoxide (DMSO) and treated to cells. DMSO was used as a control and the final DMSO concentration did not exceed 0.1% (v/v).

Cell viability assay

MTT assay was performed to evaluate cytotoxicity of acacia honey extracts. Briefly, acacia honey extracts were treated to the cells at 24 h after cell culture on a 96-well plate at a density of 3 × 103 cells/well for 24 h or 48 h. After treatment of acacia honey extracts, 50 ㎕ of MTT solution (1 ㎎/㎖) was added to the cells and incubated for 2 h. Then, cell culture supernatants were removed and DMSO was added to the cells for dissolving the resulting crystals. The formation of formazan was measured by reading absorbance at a wavelength of 570 ㎚ using UV/Visible spectrophotometer (Human Cop., Xma-3000PC, Seoul, Korea).

Measurement of NO production

Griess assay was performed to determine NO production. Briefly, each honey extract was pretreated to RAW264.7 cells for 6 h at 24 h after the cell culture on a 12-well plate at a density of 1 × 10^5 cells/well. After the treatment of each honey extract, LPS (1 ㎍/㎖) was co-treated to RAW264.7 cells for the induction of inflammatory response for 18 h. After 18 h, 100 ㎕ of the cell culture supernatants was mixed with 100 ㎕ of Griess reagent (Sigma Aldrich) at room temperature for 15 min, and then the absorbance was measured at 540 ㎚ using UV/Visible spectrophotometer (Human Cop., Xma-3000PC, Seoul, Korea).

Isolation of nucleus fraction

Nuclear fractions from RAW264.7 cells after treatment of honey extracts and LPS were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocols. Briefly, RAW264.7 cells were harvest with cold 1 × hypotonic buffer and reacted at 4℃ for 15 min. Then, detergent was added and vortexed for 10 s. RAW264.7 cells were centrifuged at 14,000 g for 1 min at 4℃ and the cell pellets were collected for the extraction of nuclear fraction. The collected pellets were lyzed using lysis buffer by being incubated at 4℃ for 30 min under shaking. After 30 min, nuclear fractions from the cell pellets were centrifuged at 14,000 g for 10 min at 4℃, and the supernatants (nuclear fraction) were stored at -80℃ for further analysis.

SDS-PAGE and Western blot

After treatment with honey extracts and LPS, RAW264.7 were washed twice with cold 1×phosphate-buffered saline (PBS) three times, and the cellular proteins were extracted

using radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich) at 4℃ for 30 min. The concentration of the cellular proteins from RAW264.7 cells was quantified using BCA protein assay (Thermo Fisher Scientific, Waltham, MA USA). The equal proteins were separated on SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The PVDF membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) by stirring at room temperature for 1 h and then incubated with specific primary antibodies in 5% non-fat dry milk in 0.05% TBS-T at 4℃ for 16 h. After 16 h, the PVDF membranes were washed three times for 5 min with 0.05% TBS-T, and then incubated with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature. Chemiluminescence was detected with ECL Western blotting substrate (Amersham Biosciences, Piscataway, NJ, USA) and visualized in Polaroid film. The density of Western blot bands was calculated using the software UN-SCAN-IT gel version 5.1 (Silk Scientific Inc. Orem, UT, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

After treatment of honey extracts and LPS, total RNA was extracted from RAW264.7 cells using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and 1 μg of total RNA was synthesized using a Verso cDNA Kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol. PCR was performed using PCR Master Mix Kit (Promega, Madison, WI, USA) and mouse primers for iNOS, COX-2, IL-1β, IL-6, TNF-α MCP-1 and GAPDH as followed : mouse iNOS: forward 5′-ttgtgcatcgacctaggctggaa-3′ and reverse 5′-gacctttcgcattagcatggaagc-3′, mouse COX-2: forward 5′-gtactggctcatgctggacga-3′ and reverse 5′-caccatacactgccaggt cagcaa-3′, mouse IL-1β: forward 5′-ggcaggcagtatcactcatt-3′ and reverse 5′-cccaaggccacaggtattt-3′, mouse IL-6: forward 5′-gaggataccactcccaacagacc-3′ and reverse 5′-aagtgcatcatcgt tgttcataca-3′, mouse TNF-α: forward 5′-tggaactggcagaagag gca-3′ and reverse 5′-tgctcctccacttggtggtt-3′ mouse MCP-1: forward 5′-gaaggaatgggtccagacat-3′ and reverse 5′-acgggtca acttcacattca-3′, GAPDH: forward 5′-ggactgtggtcatgagcccttc ca-3′ and reverse 5′-actcacggcaaattcaacggcac-3′. The PCR results were visualized using agarose gel electrophoresis. PCR reaction conditions were used: 1 cycle of (3 min at 94℃ for denaturation), 30 cycles of (30 s at 94℃ for denaturation, 30 s at 60℃ for annealing, and 30 s at 72℃ for elongation), and 1 cycle of (5 min for extension at 72℃). The density of mRNA bands was calculated using the software UN-SCAN-IT gel version 5.1 (Silk Scientific Inc. Orem, UT, USA).

Transient transfection and luciferase activity

Transient transfection for NF-κB luciferase activity was performed using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA). RAW264.7 cells (1×10^5 cells/well) were cultured on 12-well plates for 24 h and then treated with plasmid mixtures containing 1 ㎍ of the NF-κB luciferase constructs (Addgene, Cambridge, MA, USA) and 0.1 ㎍ of pRL-null vector and then cultured for 24 h. After 24 h, honey extracts were pretreated to RAW264.7 cells for 6 h, and then LPS (1 ㎍/㎖) was co-treated to RAW264.7 cells for 18 h. After treatment, RAW264.7 cells were then harvested in 1 × luciferase lysis buffer, and luciferase activity was normalized to the pRL-null luciferase activity using a dual-luciferase assay kit (Promega, Madison, WI, USA).

Statistical analysis

All experiments were performed in three replicates and the data were shown as mean ± SD (standard deviation). Statistical significance was determined by Student's *t*-test. Differences with $^{*}P$ or $^{\#}P < 0.05$ were considered statistically significant.

Results and Discussion

Inhibitory effect of acacia honey on the production of NO through suppression of iNOS expression in LPS-stimulated RAW264.7 cells

Nitric oxide (NO) has been known to play a major role in the pathogenesis of inflammation. Although proper NO exerts anti-inflammatory activity, excessive NO acts as an pro-inflammatory mediator that causes inflammation (Sharma *et al*., 2007). Thus, NO is considered to be an important target

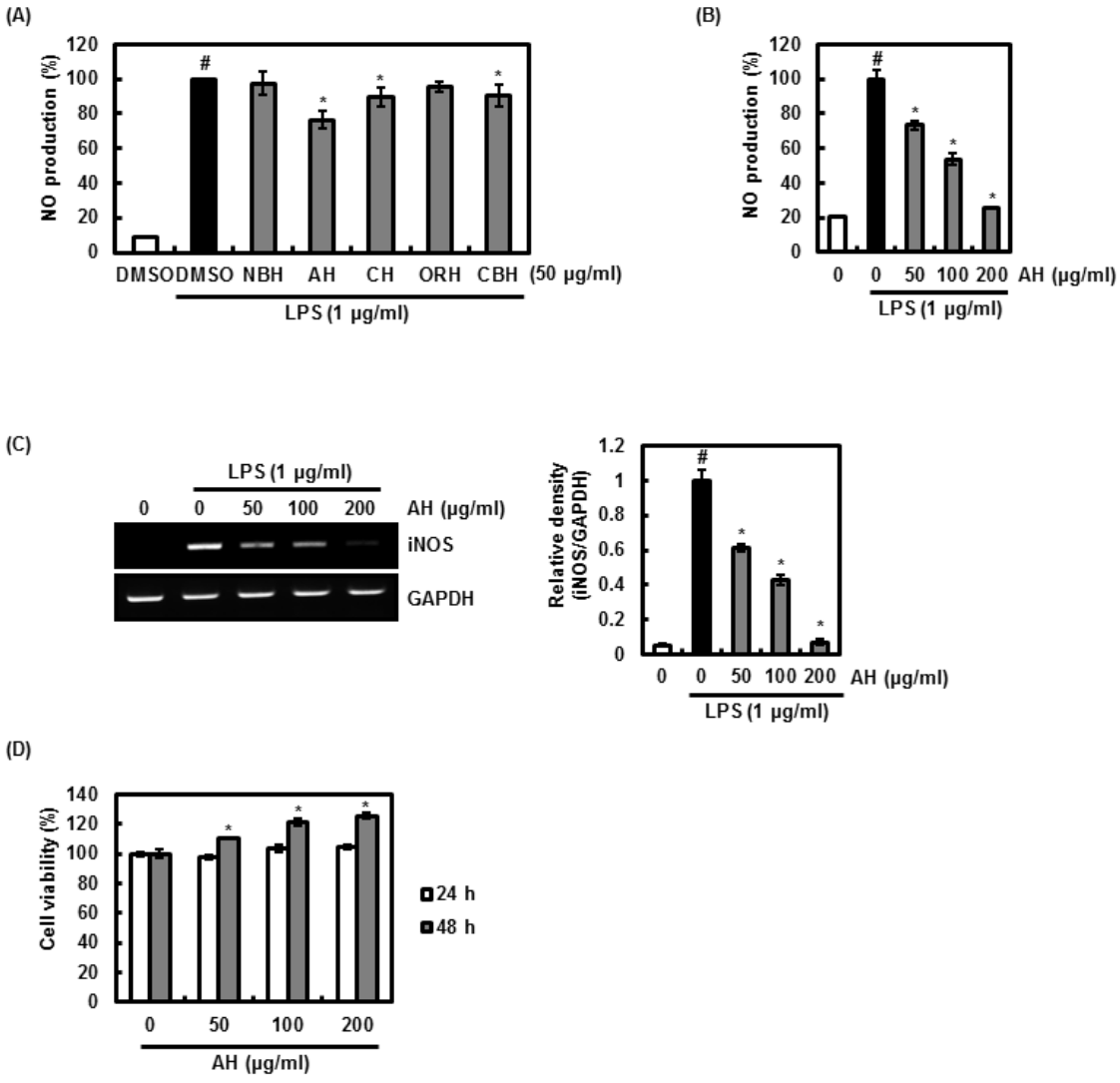

Fig. 1. AH attenuates NO production through inhibition of iNOS expression in LPS-stimulated RAW264.7 cells. (A) RAW264.7 cells were pretreated with the honey extracts according to honey plants for 6 h and then co-treated with LPS (1 ㎍/㎖) for 18 h. The determination of NO was measured by Griess assay. (B) RAW264.7 cells were pretreated with AH for 6 h and then co-treated with LPS (1㎍/㎖) for 18 h. The determination of NO was measured by Griess assay. (C) RAW264.7 cells were pretreated with AH for 6 h and then co-treated with LPS (1 ㎍/㎖) for 18 h. Total RNA was prepared after LPS and AH treatment. GAPDH was used as internal control for RT-PCR. (D) RAW264.7 cells were pretreated with AH for 24 h or 48 h. The cell viability was measured by MTT assay. $^{\#}P < 0.05$ compared to the cells without the treatment alone, and $^{*}P < 0.05$ compared to the cells treated with LPS.

for the development of therapeutic agents for the inflammatory diseases (Sharma *et al*., 2007).

It has been reported that honey contains various components such as flavonoids and phenolic compounds, and the quantity of these components contained in honey varies depending on the honey plants (Martos *et al*., 1997). So, we hypothesized that the anti-inflammatory activity of honey may be different according to the honey plants. Thus, we performed the comparative study of honey such as native bee honey (NBH), acacia honey (AH), chestnut honey (CH), oilseed rape honey (ORH) and citrus blossom honey (CBH) according to the honey plants on inhibitory effect on NO production in LPS-stimulated RAW264.7. As shown in Fig. 1A, the inhibitory effect on NO production was highest in AH, but ORH did not

inhibit NO production. Thus, AH was collected for the further study. In a dose-dependent experiment, AH significantly blocked NO production at the dose-dependent manner (Fig. 1B).

Because excessive NO is produced by inducible nitric oxide synthase (iNOS) (Korhonen *et al*., 2005), iNOS inhibitors have been used to treat NO-induced inflammatory diseases (Sharma *et al*., 2007). Thus, we investigated whether AH-mediated inhibition of NO production results from the regulation of iNOS expression in LPS-stimulated RAW264.7 cells. As shown in Fig. 1C, the treatment of LPS alone increased iNOS expression, but AH dramatically attenuated LPS-mediated overexpression of iNOS in RAW264.7 cells. To investigate the cytotoxicity of AH in RAW264.7 cells, cell viability was determined using MTT assay. The various concentrations (50, 100, 200 ㎍/㎖) of AH were treated to RAW264.7 cells for 24 h or 48 h. As shown in Fig. 1D, the cytotoxic effect of AH was not observed in RAW264.7 cells, proving that the inhibitory effect of AH against LPS-induced NO production was not its cytotoxicity. These results indicate that AH may block excessive NO production through suppression of iNOS expression in the inflammatory response.

Inhibitory effect of AH on the expression of pro-inflammatory mediators in LPS-stimulated RAW264.7 cells

To further evaluate the anti-inflammatory effect of AH, we investigated whether AH inhibits the expressions of pro-inflammatory mediators such as cyclooxygenase-2 (COX-2), interleukin (IL)-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1). COX-2 as one of the pro-inflammatory mediators is inducibly overexpressed in the inflammatory response (Shin *et al*., 2017). Thus, COX-2 inhibition has been reported to exert anti-inflammatory activity (Surh *et al*., 2001) and COX-2 has been regarded as a valuable target for developing anti-inflammatory drugs (Flower, 2003). In this study, AH did not inhibit LPS-mediated overexpression of COX-2 in RAW264.7 cells (Fig. 2).

As pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α have been known to be excessively secreted during the process of inflammation, and these cytokines contribute to the pathogenesis of the inflammatory diseases such as rheumatoid arthritis, chronic hepatitis, periodontitis and atherosclerosis (Dang *et al*., 2008). Therefore, the regulation of these cytokines has been regarded as a fundamental element for treating chronic inflammation-related disorders (Dang *et al*., 2008). In this study, LPS increased the expression of IL-1β and IL-6 in RAW264.7 cells (Fig. 2). However, AH (50-200 ㎍/㎖) dramatically attenuated LPS-mediated overexpression of IL-1β and IL-6 in RAW264.7 cells. In addition, the inhibition of TNF-α expression induced by LPS was observed in the cells treated with 200 ㎍/㎖ of AH (Fig. 2).

MCP-1 as a CC chemokine stimulating mononuclear leukocyte has been regarded as one of the pro-inflammatory chemokines (Baggiolini *et al*., 1997) and MCP-1 has been reported to be involved in the pathogenesis of inflammatory diseases such as atherosclerosis and osteoporosis (Zhong *et al*., 2013). Thus, the inhibition of MCP-1 expression has been regarded as one of the therapeutic strategies for treatment of these diseases (Zhong *et al*., 2013). In this study, AH dose-dependently attenuated LPS-mediated overexpression of MCP-1 in RAW264.7 cells (Fig. 2). From these results, AH blocked NO production through downregulation of iNOS expression and inhibited the expressions of IL-1β, IL-6, TNF-α and MCP-1, which indicated that AH may exert anti-inflammatory activity.

Inhibitory effect of acacia honey on NF-κB activation in LPS-stimulated RAW264.7 cells

Under inflammatory stimuli, IκB-α is rapidly phosphorylated by IκB kinase (IκK) complex and subsequently degraded. IκB-α degradation contributes to the translocation of NF-κB dimers (p65 and p50) from the cytoplasm to nucleus. The nuclear translocation of p65 and p50 activates NF-κB to promote transcriptional activity of pro-inflammatory mediators such as iNOS, IL-1β, IL-6, TNF-α and MCP-1 (Ducut Sigala *et al*., 2004). Thus, NF-κB has been reported to be one of the essential transcriptional factors associated with the production of pro-inflammatory mediators in the inflammatory response (Gilmore, 2006; Li and Verma, 2002; Wang *et al*., 2015). Indeed, abnormal NF-κB signaling activation by the inflammatory stimuli has been involved in the pathogenesis of

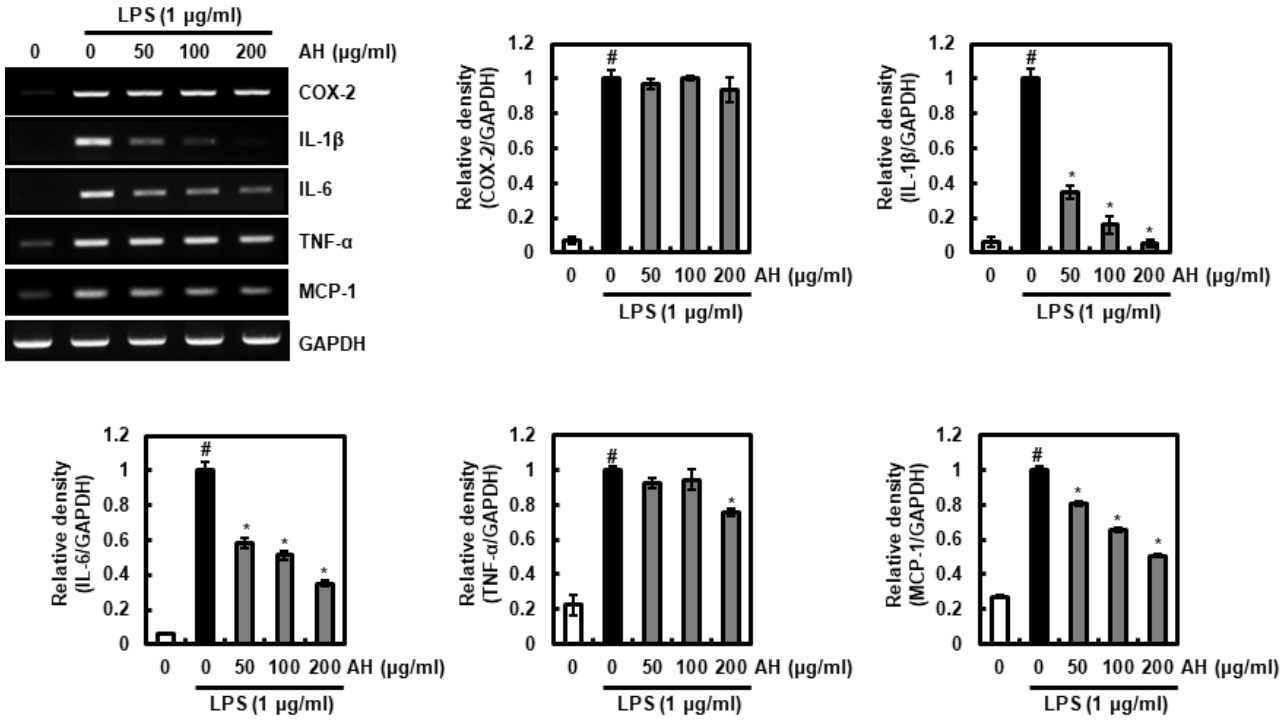

Fig. 2. AH inhibits the expression of pro-inflammatory mediators in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with AH for 6 h and then co-treated with LPS (1 ㎍/㎖) for 18 h. Total RNA was prepared after LPS and AH treatment. GAPDH was used as internal control for RT-PCR. $^{\#}P < 0.05$ compared to the cells without the treatment alone, and $^{*}P < 0.05$ compared to the cells treated with LPS.

inflammatory diseases (Abraham, 2003; Edwards *et al*., 2009). Therefore, NF-κB signaling has been known to be one of the targets for development of anti-inflammatory drugs. In present study, AH blocked LPS-mediated degradation of Iκ B-α compared to the cells treated with LPS alone (Fig. 3A). In addition, it was observed that LPS increased nuclear p65 accumulation compared to the cells without AH and LPS, but the presence of AH attenuated the level of nuclear p65 (Fig. 3B). Also, we observed that AH dose-dependently inhibited NF-κB activation (Fig. 3C). From these findings, the inhibition of NF-κB signaling may contribute to AH-mediated down-regulation of pro-inflammatory mediators.

Inhibitory effect of acacia honey on MAPK/ATF2 activation in LPS-stimulated RAW264.7 cells

In addition to NF-κB signaling pathway, MAPK is also reported to induce the expression of inflammatory mediators in the inflammatory stimuli (Guha and Mackman, 2001). Therefore, MAPK has also regarded as a typical molecular target for the development of anti-inflammatory agents (Arthur and Ley, 2013). Among the MAPK constituents such as ERK1/2, p38 and JNK, it has been reported that the inhibition of p38 or JNK has been reported to dramatically attenuate the expression of pro-inflammatory mediators, while ERK1/2 inhibition induced slight suppression of the expression of pro-inflammatory mediators (Herlaar and Brown, 1999; Hsu *et al*., 2012; Liu *et al*., 2012; Schmitt *et al*., 2007; Schuh and Pahl, 2009). In present study, AH significantly inhibited LPS-induced phosphorylation of ERK1/2 and p38 in RAW264.7 cells (Fig. 4A). However, AH did not attenuated JNK phosphorylation in LPS-stimulated RAW264.7 cells (data not shown).

Interestingly, there is growing evidence that the activation of ERK1/2 and p38 mediates the phosphorylation of ATF2 as a transcription factor, and phosphorylated ATF2 results in the nuclear accumulation of ATF2 which induce the expression of pro-inflammatory mediators (Yu *et al*., 2014). Actually, the expressions of TNF-α, IL-1β and IL-6 were dramatically

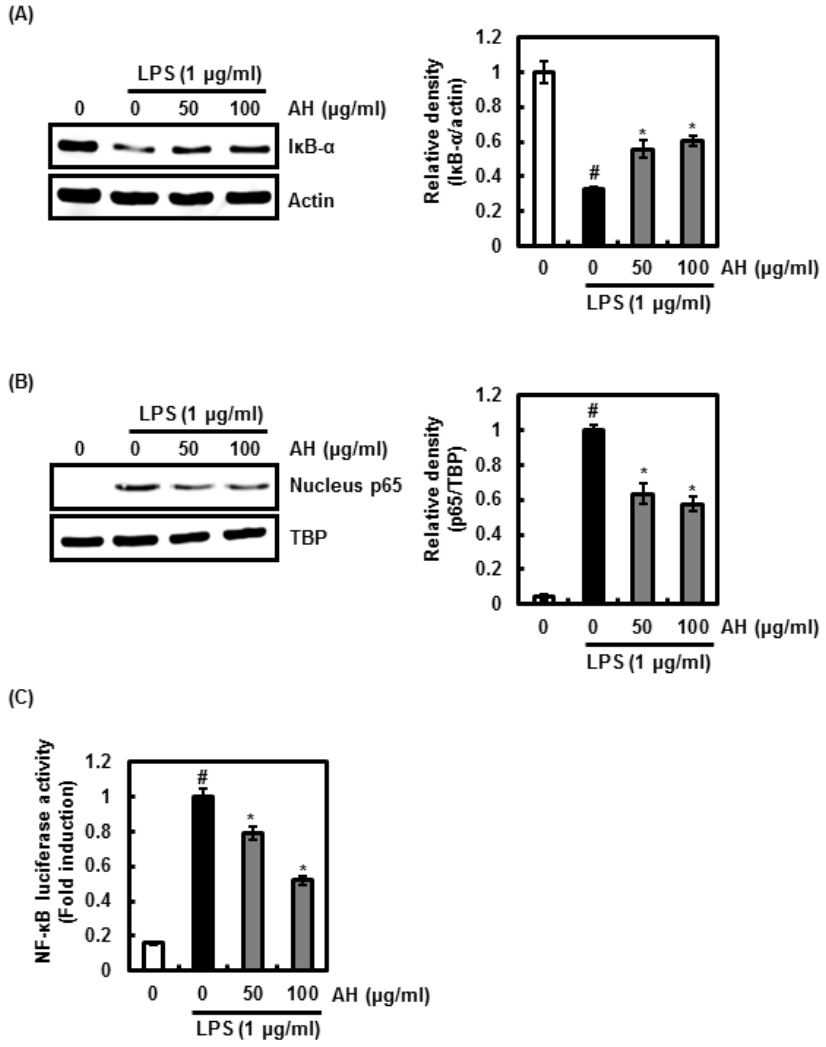

Fig. 3. AH inhibits NF-κB signaling in LPS-stimulated RAW264.7 cells. (A) RAW264.7 cells were pretreated with AH for 6 h and then co-treated with LPS (1 ㎍/㎖) for 30 min. (B) RAW264.7 cells were pretreated with AH for 6 h and then co-treated with LPS (1 ㎍/㎖) for 45 min. After the treatment, the nucleus was prepared. For Western blot analysis (A and B), the cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against IκB-α and p65. Actin and TBP were used as internal control for Western blot analysis. (C) RAW264.7 cells were co-transfected with NF-κB luciferase constructs and pRL-null. The cells were pretreated with AH for 6 h and then co-treated with LPS (1 ㎍/㎖) for 18 h. Luciferase activity for NF-κB was measured as a ratio of firefly luciferase signal/renilla luciferase signal using a dual luciferase assay kit. $^{\#}P < 0.05$ compared to the cells without the treatment alone, and $^{*}P < 0.05$ compared to the cells treated with LPS.

attenuated in ATF2-deficient mice (Reimold *et al*., 2001). Thus, ATF2 has been regarded as an attractive target for development of anti-inflammatory agents (Yu *et al*., 2014). Indeed, various natural compounds such as biochanin-A, pimaric acid, nomilin, piperine have been reported to exert anti-inflammatory activity through the inhibition of ATF2 activation (Kole *et al*., 2011; Pradeep and Kuttan, 2004; Pratheeshkumar et al., 2012; Suh *et al*., 2012). In addition, some traditional herbs such as Schizonepeta tenuifolia and HangAmDan-B have been observed to have anti-inflammatory effect through the inhibition of ATF2 (Kang *et al*., 2010; Yu *et al*., 2013). In present study, AH dose-dependently inhibited ATF2 phosphorylation (Fig. 4B) and nuclear accumulation of ATF2 (Fig. 4C). From these results, the AH-mediated-inhibition of ATF2 activation through suppression of ERK1/2 and p38 activation may contribute to AH's anti-inflammatory

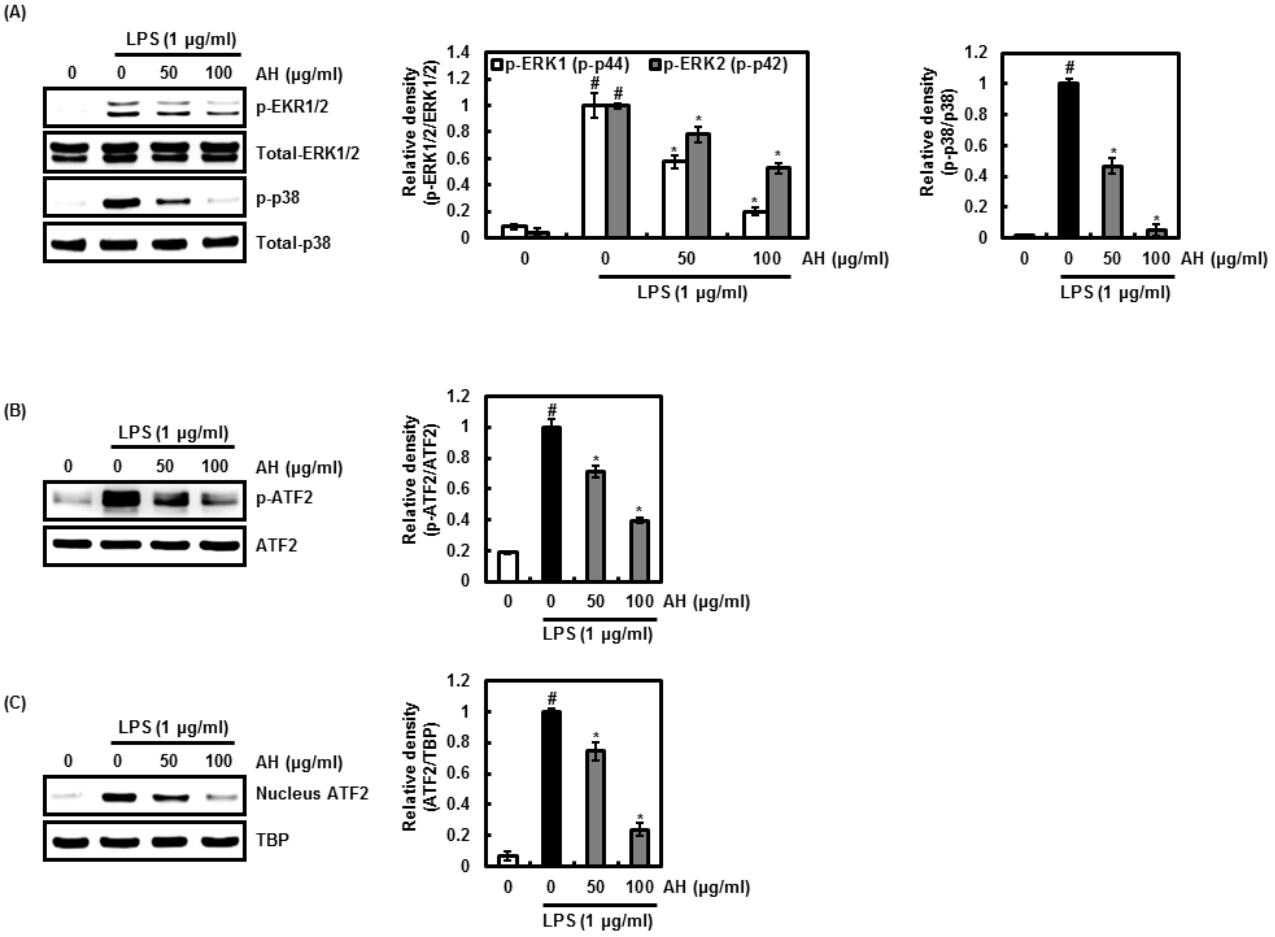

Fig. 4. AH inhibits MAPK/ATF2 signaling in LPS-stimulated RAW264.7 cells. (A and B) RAW264.7 cells were pretreated with AH for 6 h and then co-treated with LPS (1 ㎍/㎖) for 30 min. (C) RAW264.7 cells were pretreated with AH for 6 h and then co-treated with LPS (1 ㎍/㎖) for 1 h. After the treatment, the nucleus was prepared. For Western blot analysis (A, B and C), the cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against p-ERK1/2, p-p38 and p-ATF2. Total-ERK1/2, total-p38, total-ATF2 and TBP were used as internal control for Western blot analysis.

effect.

In conclusion, the current study demonstrated that acacia honey showed anti-inflammatory effect by attenuating the generation of pro-inflammatory mediators such as NO, iNOS, TNF-α, IL-6, IL-1β and MCP-1 via inhibiting NF-κB and MAPK/ATF2 signaling pathways. These findings suggest that acacia honey may have great potential for the development of anti-inflammatory drug to treat acute and chronic inflammatory disorders. However, the anti-inflammatory effect of acacia honey in vivo and the identification of major compound from acacia honey with the anti-inflammatory effect need further studies.

Acknowledgements

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