A Master's Thesis

Inhibitory effect and action mechanism of diphlorethohydroxycarmalol (DPHC) on the production of interleukin-6 in lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells

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February, 2014

LPS로 자극된 RAW 264.7에서 diphlorethohydroxycarmalol (DPHC)에 의한 IL-6 생성 억제효과 및 작용기전 연구

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Inhibitory effect and action mechanism of diphlorethohydroxycarmalol (DPHC) on the production of interleukin-6 in lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells

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(Supervised by professor **Eun-Sook Yoo**)

A thesis submitted in partial fulfillment of the requirement for the degree of master of science in Biomedicine & Drug Development

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1. Abstract

Inflammatory response is regulated and mediated by various immune cells, such as monocyte and macrophage, and especially macrophages are important players that are closely related to the initiation, propagation, and resolution of inflammation. Interleukin-6 (IL-6) is a representative pro-inflammatory cytokine generated in stimulated macrophages and may cause deleterious inflammatory states. Diphlorethohydroxycarmalol (DPHC) is a phlorotannin compound isolated from *Ishigeokamuarae*, a brown algae. This study was conducted to investigate the anti-inflammatory effect and action mechanism of DPHC in the lipopolysaccharide (LPS) stimulated RAW 264.7 murine macrophages. The experimental results showed that DPHC inhibited LPS-induced IL-6 production in a dose dependent manner at concentration of 12.5, 25, 50, and 100 μM. DPHC also suppressed the phosphorylation and the nuclear translocation of NF-κB (p65 and p50 subunits), a central signaling molecule in LPS induced inflammation process. Furthermore, the known inhibitors of NF-κB (pyrrolidinedithiocarbamate; PDTC, N-tosyl-L-phenylalaninechloromethyl ketone; TPCK, parthenolide) strongly inhibited IL-6 production in this system. However, DPHC had no or only minimal effects on the mitogen activated protein kinase pathways (JNK, ERK and P-38) and STAT1, 3, and 5 pathways activated by LPS. In conclusion, the present study demonstrates that DPHC could inhibit IL-6 production through the suppression of NF-κB activation and is suggested as a potential candidate for treating inflammatory diseases.

Keywords : Diphlorethohydroxycarmalol (DPHC), IL-6, NF-κB, LPS, inflammation

2. Introduction

Inflammation is known to be caused by various stimuli, such as bacteria, toxins, and other pathogens, which is usually accompanied with injures, irritation, infection of tissues. In addition, according to recent reports, inflammation is closely related to tumor progression. Inflammatory response is mediated by various immune cells, such as monocytes and macrophages. These inflammatory cells produce many chemokines and cytokines, which influence progression, growth regulation, and migration of tumor organ. Especially, macrophages play important roles in inflammation processes, such as initiation, propagation, and resolution (Lawrence, Willoughby & Gilroy 2002, Ben-Neriah, Karin 2011, Ariel, Timor 2013, Zhang, Mosser 2008, Coussens, Werb 2002). Activated macrophages secrete various inflammatory mediators and kill intracellular pathogens (Mosser 2003).

Cytokines are small proteins with molecular weights of 8 to 40,000 d, and are pharmacologically active proteins that have autocrine and paracrine effects. Cytokines are related to multiple biological activities in the response of various diseases, infection, and inflammation. Some cytokines are called “pro-inflammatory cytokines” or “anti-inflammatory cytokines”, because they clearly facilitate inflammation, and or suppress the activation of pro-inflammatory cytokines, such as Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2) (Dinarello 2000, Opal, DePalo 2000, Coppack 2001).

IL-6 is a pleiotropic cytokine which plays central roles in pro-inflammatory and autoimmune diseases responses, and exacerbates various inflammatory states, including sepsis, rheumatoid arthritis and inflammatory bowel disease (Hohki et al. 2010, Hack et al. 1989, Atreya et al. 2000). IL-6 is generated in the macrophage, and involved in inflammation. Especially, LPS-stimulated macrophages produce a number of inflammatory mediators, such

as IL-6, MIP-1 beta, etc through TLR receptor (Takeda, Akira 2005, Martin, Dorf 1991). According to Fattori.E. and Cappelletti. M. et al, in the absence of IL-6, mice could not be mounted a normal inflammation to tissue damage by turpentine injection. According to the results, they concluded that IL-6 is essential mediator of inflammation (Fattori et al. 1994). Therefore, IL-6 is an important factor in preventing and treating inflammation.

TLR is a trans-membrane receptor that is one of critical components in the innate immune system and autoimmunization, and directly recognize and activate immune cells. TLR signaling pathway modulates Toll-interleukin 1 receptor (TIR) domain-containing adaptor proteins included myeloid differentiation primary response gene 88 (MyD88), TIR domain containing adaptor protein (TIRAP), and TIR-domain containing adapter-inducing interferon-β (TRIF). TLRs also recognize various pathogens and participate in cell signaling pathway (Akira, Takeda 2004, Takeda, Akira 2004, Aderem, Ulevitch 2000, Akira, Uematsu & Takeuchi 2006, Kawai, Akira 2010). TLR4, a member of TLRs family, recognizes lipopolysaccharide (LPS, endotoxin) and mediates LPS-induced inflammation (Hoshino et al. 1999, Bortoluci, Medzhitov 2010). LPS, an inducer of the innate immune response, binds to the Myeloid differentiation factor-2 (MD-2)/TLR4 complex, initiates downstream signaling pathway, and releases various inflammatory chemokines and cytokines including IL-6, inducible nitric oxide synthase (iNOS), TNF-α (Akira, Takeda 2004, Park et al. 2009, Kagan, Medzhitov 2006). In the downstream signaling pathway, intracellular adaptor proteins leads to two distinct signaling pathways, MyD88-dependent pathway that includes the mitogen-activated protein kinases (MAPK) [c-Jun N-terminal kinase (JNK), p38 and extracellular-signal related kinase (ERK)] and the NF-kappaB (NF-κB; p50/p65) complex, and MyD88-independent signaling pathway that produce type 1 interferons. These type 1 interferons regulate STAT1-containing transcription factors. The STAT3 and STAT5 are also induced by LPS treatments (Lu, Yeh & Ohashi 2008, Kawai, Akira 2006, Ohmori, Hamilton 2001b, Bode, Ehlting & Haussinger 2012, Yamaoka et al. 1998).

NF-κB is one of the most principal transcription factors which involves in gene induction of cellular proliferation. NF-κB is also a pathogen-derived inflammatory cytokine that serves as a central inflammatory mediator (Akira, Takeda 2004, Park et al. 2009, Kagan, Medzhitov 2006, Lu, Yeh & Ohashi 2008, Kawai, Akira 2006, Li, Verma 2002). When the stimulus is applied, NF-κB is activated through the activation of I kappa B-kinase (IKK) complex, which phosphorylates I kappa B-alpha (IκB-α) and p50/p65. Then IκB-α undergoes proteasomal degradation. NF-κB exists mainly as a heterodimer including the subunits p50/p65 of Rel family. The p65 transactivation domain is activated by phosphorylation, and then the phosphorylation, ubiquitination, and degradation of IκB-α release the p50/p65 heterodimer. The free p50/p65 complex then translocate from cytosol to nucleus, and finally controls promotor region of target genes, which induces various pro-inflammatory factors. According to Pan, Lai et al, the expression of iNOS and COX-2 is mediated via NF-κB in LPS-stimulated macrophage (Gloire, Legrand-Poels & Piette 2006, Baeuerle, Baltimore 1996, Aggarwal 2004, Pan et al. 2006).

The cytokine-activated Janus kinase (JAK)-signaling transducer and activator of transcription (STAT) pathway has an important role in immune system. JAK-STAT signaling pathway regulates the initiation, propagation, and inflammation in all cell types. The phosphorylation of STATs is divided into two types, tyrosine phosphorylation and serine phosphorylation. The tyrosine phosphorylation acts a switching signal to activate STATs that require for dimerization, nuclear translocation and DNA binding. The serine phosphorylation seems to be independent on the tyrosine phosphorylation (Shuai, Liu 2003, O'Shea, Murray 2008). Although JAK2 induced the phosphorylation of STAT1 tyrosine and STAT1 serine, STAT1 serine phosphorylation occurs independently of tyrosine phosphorylation. STAT1 serine 727 phosphorylation is induced STAT1-mediated gene activation to the max and IFN-gamma-induced transcriptional activity of STAT1 (Zhu et al. 1997, Shuai 2003, Decker, Kovarik 2000, Wen, Zhong & Darnell 1995). The phosphorylation of STAT1-S727A, STAT1

transactivation, domain is induced IFN-gamma-dependent genes and related to innate immunity (Varinou et al. 2003). In addition to, IL-6 is an obligatory role that regulates through the JAK-STAT signal pathway, the ensuing up-regulation of iNOS and COX-2, and the development of a cardioprotective phenotype (O'Shea, Murray 2008, Kalinski 2012, Dawn et al. 2004).

Diphlorethohydroxycarmalol (DPHC) is a phlorotannin compound isolated from *Ishige okamuarae*, a brown algae, collected from the coast of Jeju island. According to recent reports, DPHC has been determined to have various biological effects. DPHC alleviated both the increase of postprandial blood glucose levels in diabetic mice and induced factors of high glucose-induced-oxidative stress in human umbilical vein endothelial cells (Heo et al. 2009, Heo et al. 2010). Also, DPHC suppressed intracellular reactive oxygen species (ROS) induced by ultraviolet (UV)-B radiation, and had protective effects on DNA tail and cell damages (Heo et al. 2010). However, little information is available regarding the ability of DPHC on the inhibition of IL-6 production.

In the present study, we examined the inhibitory effect of DPHC on the production of IL-6, a pivotal cytokine in the inflammatory process and explored the action mechanism in LPS-stimulated RAW264.7 cells.

3. Materials and methods

3.1 Reagents (Chemicals and antibodies)

Lipopolysaccharide (LPS, *E. coli* 0111:B4) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen-GIBCO (Grand Island, NY, USA). Mouse IL-6 Duoset enzyme-linked immunosorbent assay (ELISA) kits and prostaglandin E_2 parameter assay kit were obtained from R&D Systems (St. Louis, MO, USA). MAPKs (anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-JNK, anti-JNK), STATs [anti-Phospho-STAT1 (Tyr701, Ser727), anti-Phospho-STAT3 (Tyr705), anti-Phospho-STAT5 (Tyr694), anti-STAT3, anti-STAT5)], NF-κB [anti-phospho-p65 (Ser536), anti-p65, anti-IκB-α], phospho-p38 MAP kinase (Thr180/Tyr182), and anti-p38 were purchased from Cell Signaling Technology (Beverly, MA, USA). NF-κB [anti-p50 (E-10)] was purchased from Santa Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-STAT1was purchased from Becton Dickison (San Diego, CA, USA). β-actin was obtained from Sigma (St. Louis, MO, USA). DyLight 488 conjugated Donkey anti-Rabbit antibody was purchased from BioLegend Inc. (San Diego, CA, USA). All other reagents were reagent grade.

3.*2 Isolation of DPHC*

The shade dried whole plant of *Ishige okamurae* (800 g) was percolated with 70% aqueous ethanol under stirring at room temperature for 2 days. The filtrate was concentrated in a vacuum freeze-dryer, suspended in distilled water and partitioned with ethyl acetate. The ethyl acetate fraction was chromatographed over reversed phase silica gel with gradient solvent (H_2O/MeOH) system to provide 4 fractions (1~4). The fraction 1 was chromatographed over Sephadex-LH 20 with $CHCl_3$/MeOH (1/1) to provide 6 fractions (4-1'~4-6'). DPHC was obtained from the fraction 4-5', and its structure (Fig. 1) was confirmed by comparing the NMR spectral data with literatures (Heo et al. 2008, Zou et al. 2008).

3.3 Cell Culture

RAW 264.7 cells, the murine macrophage cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in DMEM supplement with 10% (vol/vol) FBS, containing100 U/ml penicillin-streptomycin (GIBCO, Grand Island, NY, USA), and were maintained at subconfluence in 5% CO_2 humidfied atmosphere at 37℃. Cells were counted with a hemocytometer.

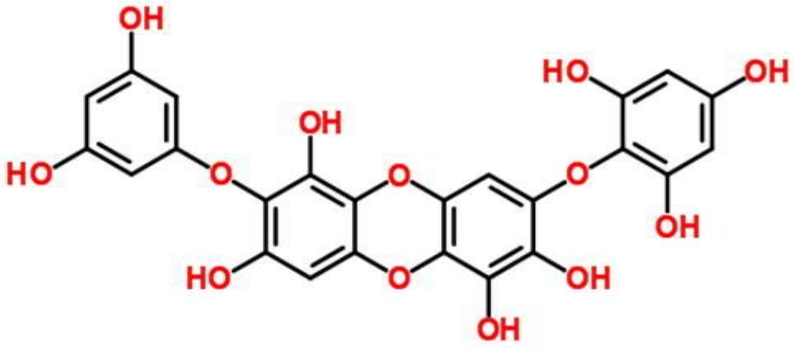

Figure 1. Chemical structure of diphlorethohydroxycarmalol (DPHC)

3.4 Cell viability

Cell viability was determined by using EZ-CyTox (tetrazolium salts, WST-1) assays (Daeil Lab Inc, Seoul, Korea). RAW 264.7 cells were seeded at a density of $1.5x10^5$ cells/ml in 96-well culture plates and were maintained for 18 h an incubator. Culture medium was replaced by new medium, DMEM supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. Cells were treated with LPS in the absence or presence of various concentrations of DPHC. After incubation for 24 h, 100 μL of culture supernatant from each well was abandoned and each well was treated with 5μL WST, which was further incubated for 2 h at 37℃ and 5% CO_2 atmosphere. After the incubation, the light absorbance of each well was quantified using a VersaMax ELISA microplate reader (Molecular Devices, CA, USA) at 450 nm.

3.5 The enzyme-linked immunosorbent assay (ELISA)

IL-6 production in the supernatant was measured by ELISA kit according to the manufacturer's instructions. Briefly, 100 μL of cell culture supernatants or standards in reagent diluent was added to each well of ELISA kit well plate and incubated for 2 h at room temperature. After washing, 100 μL of the detection antibody in reagent diluent was added to each well and incubated for 2 h at room temperature. After washing, 100 μL of streptavidin conjugated horseradish-peroxidase (HRP) was added and left at room temperature for 20 min. After washing, 100 μL of substrate solution was added to each well and left in dark place at room temperature for 20 min. Then 50 μL of stop solution (2 N sulfuric acid) was added. After the reaction was stopped, the optical density of each well was determined using a VersaMax ELISA microplate reader at 450 nm.

3.6 Western blot analysis

RAW 264.7 cells were treated to LPS with absence or presence of the elapsed concentration of DPHC. After the elapsed times of incubation, the cells were washed twice with cold phosphate buffered saline (PBS) and were lysed in a protein lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml a protinin, and 25 μg/ml leupeptin], and then the cells were collected. Then The cell lysates were isolated using centrifugation at 15,000 rpm (4℃ for 15 min). The protein concentration in the supernatants was measured by the Bradford assay (Bio-rad, Herculers, CA, USA) and all proteins were adjusted to equal protein content. Aliquots of the lysates (20~30 μg protein/lane) were separated on a NuPAGE 4-12% bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and the separated proteins were transferred from the electrophoresis gel to the surface of polyvinylidenedifluoride (PVDF) membranes using an iBlot gel transfer device (Invitrogen). Then membranes were treated with 5% non-fat skim milk as a blocking solution, and incubated with primary antibodies (1:1,000) at 4℃ overnight. After incubating, the membranes were washed by Tween 20-Tris-buffered saline (TBST) several times and incubated with secondary HRP-linked anti-rabbit or anti-mouse IgG (1:5,000, Cell signaling, Santa Cruz Biotechnology; respectively) for 90 min at room temperature. After washing, immunoactive proteins were detected by the WEST-ZOL (plus) Western Blot Detection System (iNtRON Biotechnology, Gyeonggi, Korea).

3.7 Confocal laser scanning microscopy analysis

RAW 264.7 cells were plated onto coverslips at 6-well plate and incubated for 18 h, and then treated with LPS with the absence or presence of DPHC for various times. Then the cells were washed in PBS and fixed with 3.5% formaldehyde (PFA) in PBS for 30 min at room temperature. After washing with PBS, cells were treated with 0.1M glycine for 15 min and were permeabilized with PBS containing 0.1% Triton X-100 for 10 min. After several washings with PBS, cells were blocked in PBS containing 3% BSA and 0.1% Triton X-100. In order to react cells and antibody, primary antibody anti-NF-κB (phospho-p65, p50) diluted 1:200 was treated at 4℃, overnight. Then cells were washed with Tween 20-PBS (PBST), and treated with DyLight488 conjugated donkey anti-rabbit secondary antibody diluted 1:200, and then left at dark place and room temperature for 30 min. After several washing, coverslips were mounted to slides by using VECTASHIELD Mounting Media with DAPI (Vector Labs, Burlingame, CA, USA). The images were visualized using FV500 confocal microscopy (Olympus, Tokyo, Japan).

3.8 Statistical analysis

Statistical differences between the test and control groups were analyzed by Student's t-test. Statistical results obtained from at least three independent experiments were presented as the mean and standard deviation (means ± SD). *P-value < 0.05, <0.01, and <0.001* was considered to be statistically significant.

4. Results

4.1. Anti-inflammatory effect of DPHC

4.1.1. Effect of DPHC on cell viability in LPS-stimulated RAW 264.7 cells

We measured the cell viability of LPS-induced RAW 264.7 cells at various concentrations (12.5, 25, 50, and 100 μM) of DPHC by the WST assay to exclude the possible suppression of inflammatory chemokine production by cytotoxic activity. RAW 264.7 cells were treated with various concentrations (12.5, 25, 50, and 100 μM) of DPHC for 24 h. As shown in Fig. 2, DPHC exhibited no effects on the cell viability.

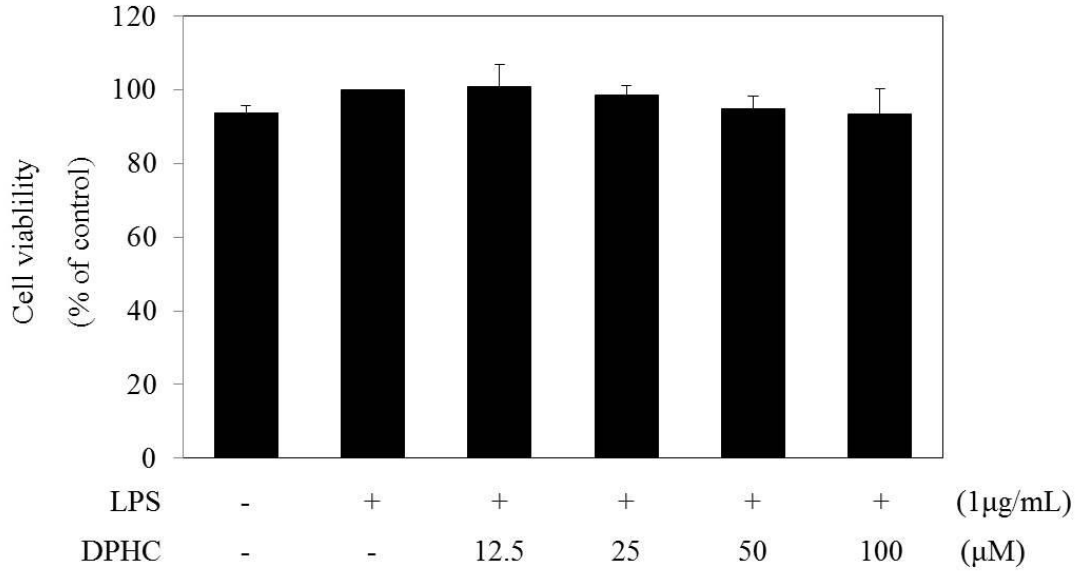

Figure 2. Effect of diphlorethohydroxycarmalol (DPHC) on the cell viability in the LPS-stimulated RAW264.7 murine macrophages.

Cells (1.5 x 10^5 cells/mL) were pre-incubated for 18 h. Cell viability was determined from the cells stimulated with LPS (1 μg/mL) in the presence or absence of DPHC for 24 h (12.5 to 100 μM). Cell viability was determined by the WST assay, and the results were expressed as percentage of surviving cells over positive cells (only LPS addition). Error bars indicates the mean ± S.D.

4.1.2.Time-course production of NO and inflammatory cytokines in LPS-stimulated RAW 264.7 cells

We measured the amount of NO and inflammatory cytokines at indicated times (0, 5, 15, 30, 60, and 120 min and 4, 6, 8, 10, 12, and 24 h) after LPS (1 μg/mL) treatment to characterize the time profile of NO and inflammatory cytokine production. NO production continuously increased from 6 h (0.030 μM) to 24 h (0.264 μM) after LPS-stimulation (Fig. 3). PGE_2production continuously increased from 6 h (11,856.46 pg/mL) to 24 h (42,724.96 pg/mL) after LPS-stimulation (Fig. 4). IL-6 production continuously increased from 6 h (312.4 pg/mL) to 24 h (918.3 pg/mL) after LPS-stimulation (Fig. 5), and TNF-α production increased from 2 h (429.4 pg/mL) to 24 h (1,147.2 pg/mL) after LPS-stimulation (Fig. 6).

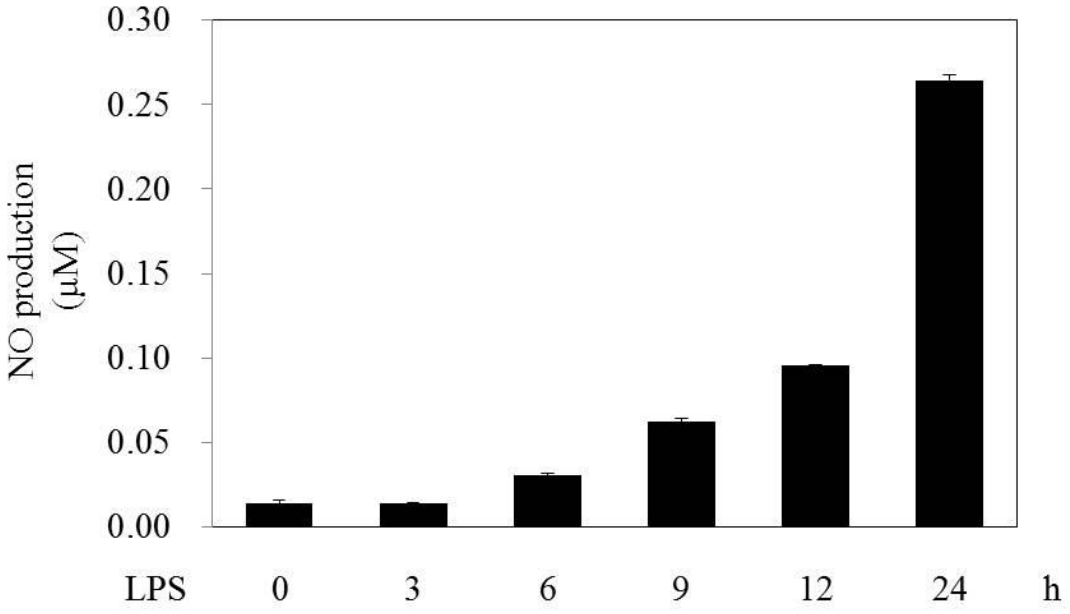

Figure 3. Nitric oxide (NO) production in LPS-stimulated RAW264.7 macrophages.

Cells (1.5×10^5 cells/mL) were pre-incubated for 18 h, and cells were stimulated with LPS (1 μg/mL) for indicated times, respectively. NO production was determined from the culture supernatants of cells. The amount of NO was determined by using Griess method. The standard curve created by $NaNO_2$ in culture medium. The measurement of NO was performed in triplicate. The error bars indicate standard deviation.

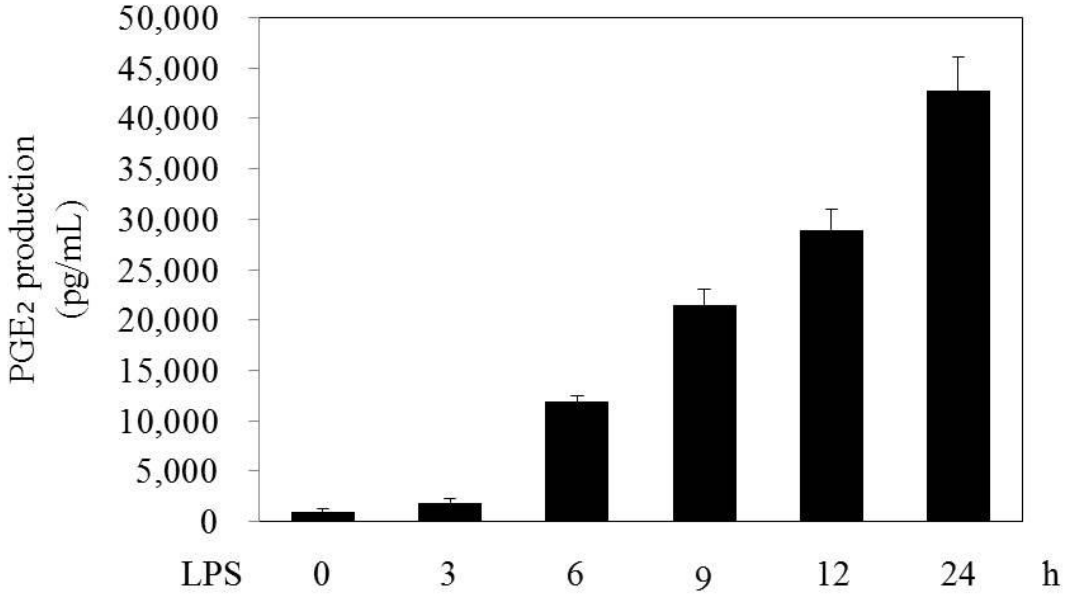

Figure 4. PGE_2 production in LPS-stimulated RAW264.7 macrophages.

Cells (1.5×10^5 cells/mL) were pre-incubated for 18 h, The cells were stimulated with LPS (1 μg/mL) for indicated times, respectively. The PGE_2 production was determined from the culture supernatants of cells. The production of PGE_2 was determined by ELISA. The measurement of PGE_2 was performed in triplicate. The error bars indicate standard deviation.

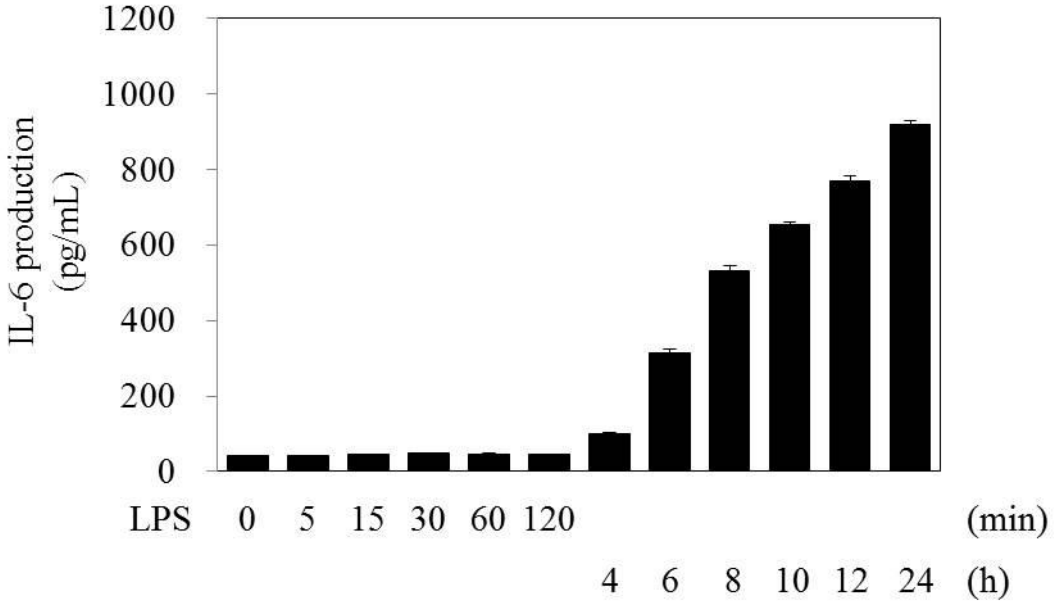

Figure 5. The production of IL-6 in LPS-stimulated RAW264.7 macrophages.

Cells (1.5×10^5 cells/mL) were pre-incubated for 18 h, and cells were stimulated with LPS (1 μg/mL) for different times, respectively. The IL-6 production was determined from the culture supernatants of cells. IL-6 production was determined by ELISA. The measurement of IL-6 was performed in triplicate. The error bars indicate standard deviation.

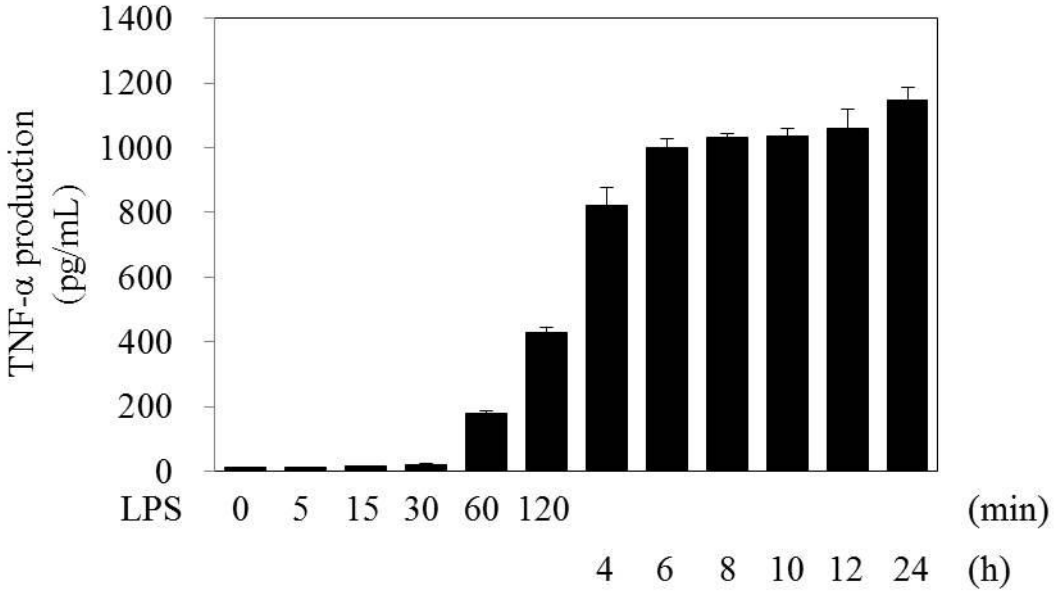

Figure 6. The production of TNF-α in LPS-stimulated RAW264.7 macrophages.

Cells (1.5×10^5 cells/mL) were pre-incubated for 18 h, cells were stimulated with LPS (1 μg/mL) for different times, respectively. TNF-α production was determined from the culture supernatants of cells. TNF-α production was determined by ELISA. The measurement of TNF-α was performed in triplicate. The error bars indicate standard deviation.

4.1.3. Effect of DPHC on NO and PGE_2 production in LPS-stimulated RAW 264.7 cells

NO plays many regulatory functions in the development of inflammation and is synthesized from many cell types with inflammation (Coleman 2001, Esposito, Cuzzocrea 2007). NO production gradually increased with the indicated times after LPS treatment, and then reached a peak at 24 h (Fig. 3). We then confirmed the production of NO after 24 h of DPHC treatment. We examined NO production by Griess assay at 24 h to determine whether or not DPHC inhibited LPS-stimulated NO production. As shown in Fig. 7, LPS induced NO production. After 24 h of treatment, DPHC weakly reduced NO production in LPS-stimulated RAW 264.7 cells, and this result was no statistically significant. PGE_2 also involves inflammatory mediators from LPS-stimulated resident macrophages, and the PGE_2-EP4 signaling pathway promotes immune inflammation (Dieter et al. 1999, Yao et al. 2009). PGE_2 production gradually increased with the elapsed times after LPS treatment and then reached a peak at 24 h (Fig. 3). We then confirmed the production of PGE_2 after 24 h of DPHC treatment. We examined PGE_2production by ELISA to investigate whether or not DPHC inhibited LPS-stimulated PGE_2 production. DPHC significantly reduced PGE_2 production in a dose-dependent manner for 24 h in LPS-stimulated RAW 264.7 cells (Fig. 8).

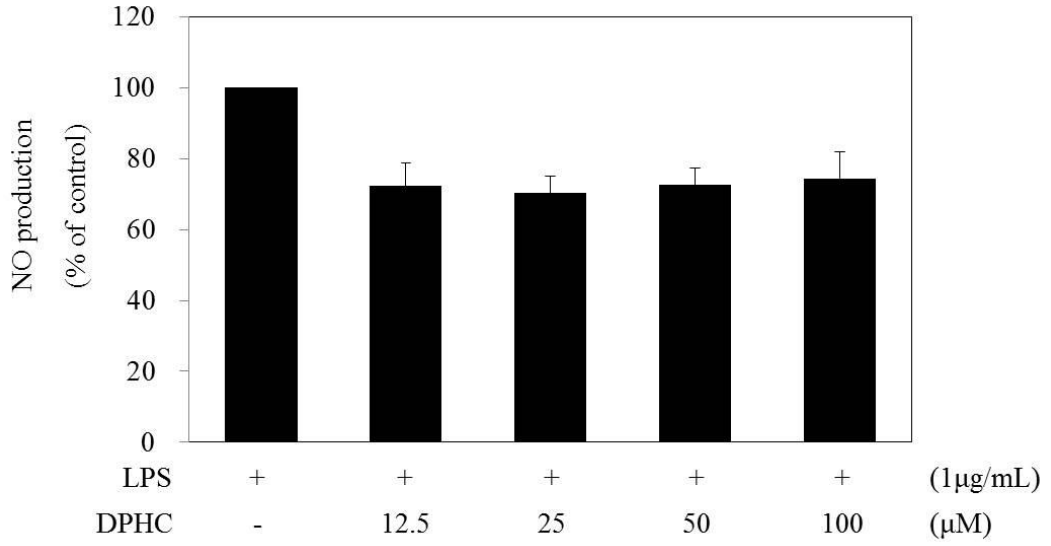

Figure 7. Effect of diphlorethohydroxycarmalol (DPHC) on the nitric oxide (NO) production in the LPS-stimulated RAW264.7 murine macrophages.

Cells (1.5 x 10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 h and then NO production was determined from the culture supernatants of cells stimulated with LPS (1 μg/mL) in the presence of DPHC (12.5, 25, 50 , 100 μM) for 24 h. NO production was determined by the Griess reagent method. The measurement of NO was done in triplicate. Error bars indicate ± S.D.

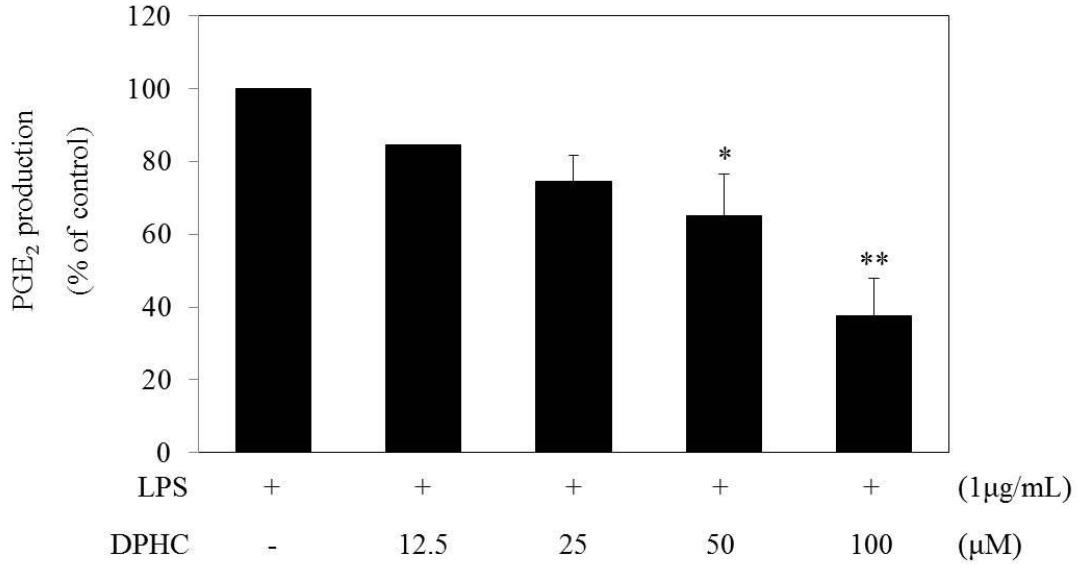

Figure 8. Effect of diphlorethohydroxycarmalol (DPHC) on the Prostaglandin E_2 (PGE_2) production in the LPS-stimulated RAW264.7 murine macrophages.

Cells (1.5 x 10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 h and then PGE_2production was determined from the culture supernatants of cells stimulated with LPS (1 μg/mL) in the presence of DPHC (12.5, 25, 50, 100 μM) for 24 h. PGE_2 production was determined by ELISA. The measurement of PGE_2 was performed in duplicate, and the results were expressed as percentage over positive cells. Error bars indicate ± S.D. *, P<0.05, **, P<0.01, significant when compared with LPS positive control.

4.1.4. Effect of DPHC on IL-6 and TNF-α production in LPS stimulated RAW 264.7 cells

Endotoxin produces pro-inflammatory cytokines, such as IL-6 and TNF-α (Carlson et al. 1999). IL-6 mediate inflammation, infection, and metabolism, whereas TNF-α activates inflammatory and toxic cytokines (Scheller et al. 2011, Aggarwal 2003). IL-6 production gradually increased at 6 h and peaked at 24 h, and TNF-α production gradually increased from 1 h to 4~6 h and then peaked at 24 h (Figs. 5 and 6). We then confirmed the production of PGE_2 after 24 h of DPHC treatment. We examined IL-6 and TNF-α production by ELISA for 6 h and 24 h, respectively, to determine whether or not DPHC inhibited the production of IL-6 and TNF-α. RAW 264.7 cells were treated with LPS with and without DPHC for 6 and 24 h, respectively. DPHC did not reduce TNF-α production in LPS-stimulated RAW 264.7 cells (Figs. 9A and 9B). DPHC inhibited IL-6 production in a dose-dependent manner at 6 and 24 h (Figs. 10A and 10B). The significant effect of DPHC after 24 h incubation is shown in Fig. 10A. The results suggest that DPHC primarily regulates the accumulation of IL-6 as a pro-inflammatory cytokine.

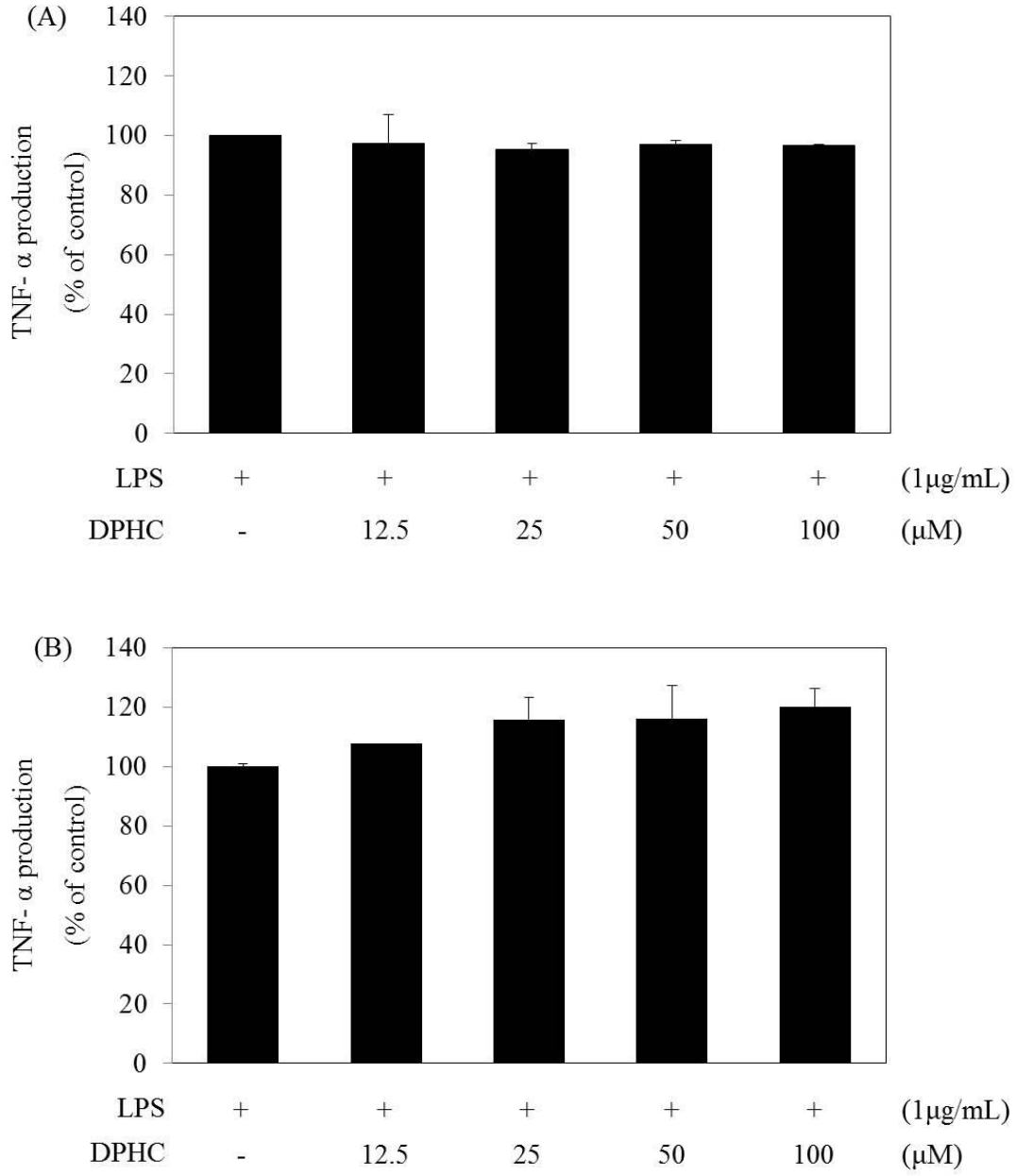

Figure 9. Effect of diphlorethohydroxycarmalol (DPHC) on the Tumor necrosis factor-α (TNF- α) production in the LPS-stimulated RAW264.7 murine macrophages.

(A) Cells (1.5 x 10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 h and then TNF-α production was determined from the culture supernatants of cells stimulated with LPS (1 μg/mL) in the presence of DPHC (12.5, 25, 50, 100 μM) for 24 h. TNF-α production was determined by ELISA. The measurement of TNF-α was done in duplicate, and the

results were expressed as percentage over positive cells. Error bars indicate ± S.D. (B) Cells (1.5 x 10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 h and then TNF-α production was determined from the culture supernatants of cells stimulated with LPS (1 μg/mL) in the presence of DPHC (12.5, 25, 50, 100 μM) for 6 h. TNF-α production was determined by ELISA. The measurement of TNF-α was performed in triplicate, and the results were expressed as percentage over positive cells. Error bars indicate ± S.D.

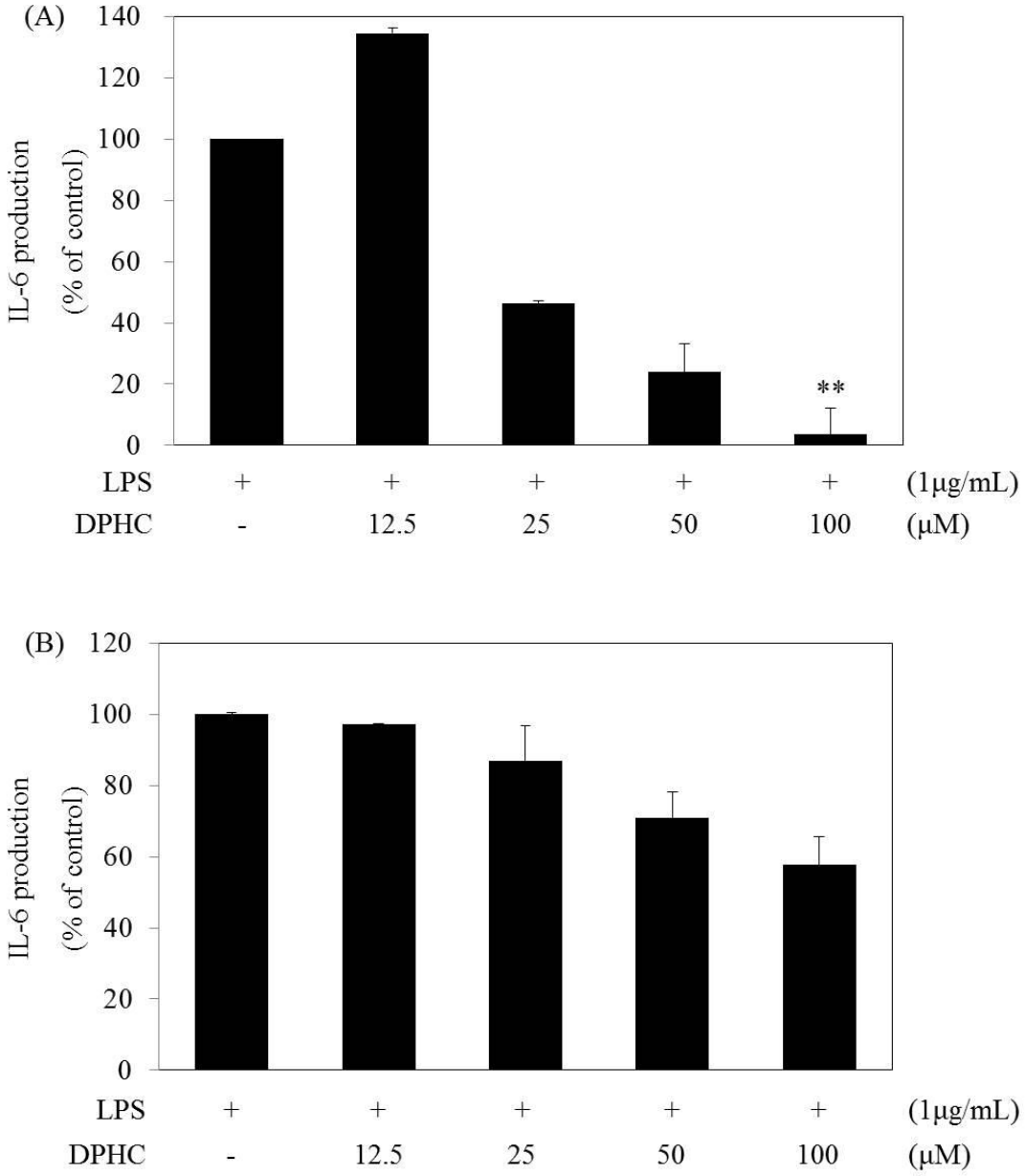

Figure 10. Effect of diphlorethohydroxycarmalol (DPHC) on the Interleukin-6 (IL-6) production in the LPS-stimulated RAW264.7 murine macrophages.

(A) Cells (1.5 x 10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 h and then IL-6 production was determined from the culture supernatants of cells stimulated with LPS (1 μg/mL) in the presence of DPHC (12.5, 25, 50, 100 μM) for 24 h. IL-6 production was determined by ELISA. The measurement of IL-6 was done in duplicate, and the results were expressed as percentage over positive cells. Error bars indicate ± S.D. **, P<0.01, significant

when compared with LPS positive control. (B) Cells (1.5 x 10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 h and then IL-6 production was determined from the culture supernatants of cells stimulated with LPS (1 μg/mL) in the presence of DPHC (12.5, 25, 50, 100 μM) for 6 h. IL-6 production was determined by ELISA. The measurement of IL-6 was performed in duplicate, and the results were expressed as percentage over positive cells. Error bars indicate ± S.D.

4.2 . Effect of DPHC on the signaling pathway of LPS

4.2.1. Effect of DPHC on iNOS and COX-2 expression and production in LPS-stimulated RAW 264.7 cells

Endotoxin (bacterial lipopolysaccharide) is the product of inducible NO synthase (iNOS) and inducible cyclooxygenase (COX-2) in monocytes and macrophages. iNOS and COX-2 disrupt the inflammatory mechanism in inflammatory cells (Murakami, Ohigashi 2007, Lazarov, Balutsov & Ianev 2000). Therefore, we therefore examined the effect of DPHC on the LPS-induced activity of iNOS and COX-2 at each time course (0, 5, 15, 30, 60, 120, 240, and 360 min) by western blot. When the RAW 264.7 cells were stimulated by LPS (1 μg/mL) for each time course (0, 5, 15, 30, 60, 120, 240, and 360min and 0, 3, 6, 9, 12, and 24 h), the expression levels of iNOS and COX-2began to increased at 360 min and then peaked at 24 h after LPS stimulation (data not shown). We used western blot to determine whether or not DPHC is affected by the down-regulation of iNOS and COX-2 protein levels. Various concentrations (12.5, 25, 50, and 100 μM) of DPHC lightly inhibited iNOS expression in the LPS-stimulated RAW 264.7 cells, and 100 μM of DPHC lightly inhibited COX expression in these cells (Fig. 11). These results suggest that DPHC slightly inhibits the expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells.

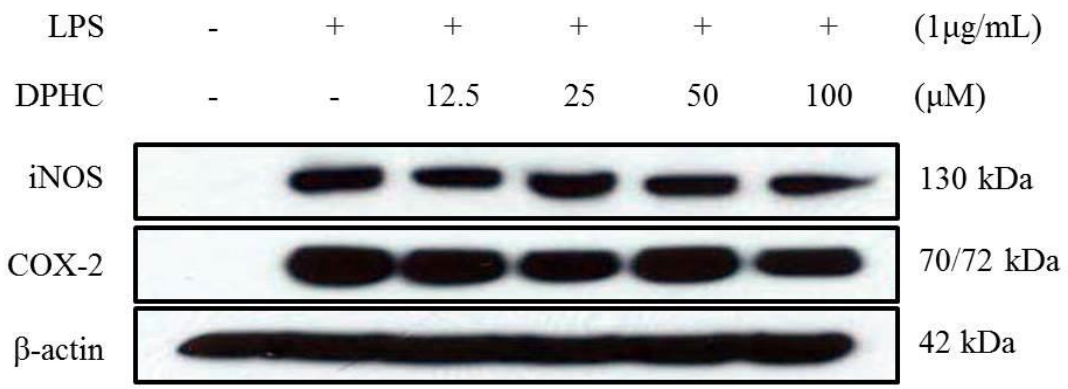

Figure 11. Effect of diphlorethohydroxycarmalol (DPHC) on Expression of iNOS and COX-2 in LPS-stimulated RAW264.7 murine macrophages.

RAW264.7 cells (7.5×10^5 cells/mL) were pre-incubated for 18 h. And cell were stimulated with LPS (1 μg/mL) and treated presence or absence of DPHC (12.5 to 100 μM) for 24 h. Then, expression of iNOS, COX-2 and β-actin proteins was determined by Western blotting of whole cell lysates with the indicated antibodies.

4.2.2. Effect of signaling inhibitors on IL-6 production in LPS-stimulated RAW 264.7 cells

We examined IL-6 production in the signaling inhibitors of mitogen-activated protein kinases (MAPKs; SP600125, PD098059, and SB203580) by ELISA to determine whether or not IL-6 production involves the generating mechanism (Assi et al. 2006, Alessi et al. 1995, Badger et al. 1996). Compared with the positive group of treated LPS, SP and SB 203580 were involved in IL-6 production (Fig. 12B).

Aside from the MAPK inhibitors, we examined whether or not STAT inhibitor suppresses IL-6 production. We used STAT inhibitors AG490, (-)-epigallocatechingallate (EGCG), and fludarabine. AG490 is Janus kinase (JAK) inhibitor, EGCG is a STAT inhibitor, and fludarabine is a STAT-1 inhibitor (Seo et al. 2009, Tedeschi, Suzuki & Menegazzi 2002, Frank, Mahajan & Ritz 1999). In this study, compared with the positive group of treated LPS, AG490 and JI were involved in IL-6 production (Fig. 12C).

We examined IL-6 production in the signaling inhibitors of NF-κB (pyrrolidinedithiocarbamate, PDTC; N-tosyl-L-phenylalaninechloromethyl ketone, TPCK; parthenolide) by ELISA to determine whether or not IL-6 production involves the generating mechanism. PDTC, TPCK, and parthenolide are strong NF-κB inhibitors (Wang et al. 2011, Ha et al. 2009, Saadane et al. 2007). Compared with the positive group of treated LPS, NF-κB inhibitors significantly reduced IL-6 production. NF-κB is relevant to IL-6 production (Fig. 12A). However, compared with the result shown in Fig. 2A, the results in Fig. 12 showed that DPHC did not inhibit of MAPK mechanism but inhibited NF-κB mechanism. These results suggest that DPHC inhibits IL-6 production via the NF-κB signaling pathway.

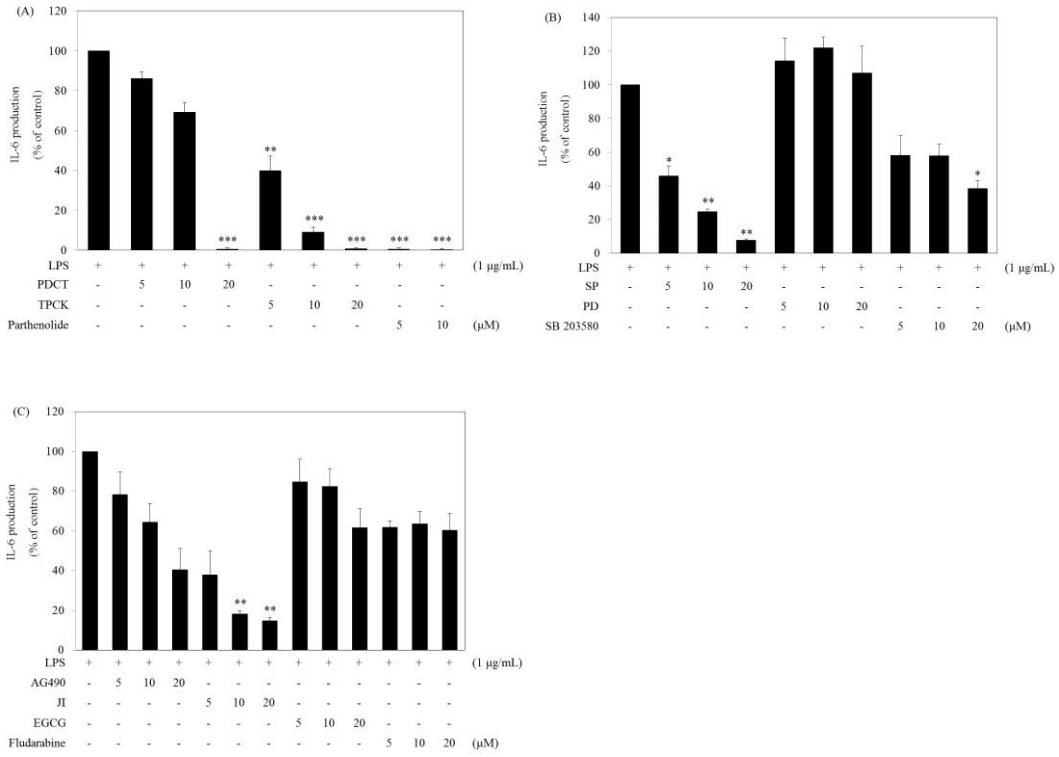

Figure 12. Effect of NF-κB inhibitors (PDCT, TPCK, parthenolide), MAPK inhibitors (SP, PD, SB 203580), and STAT inhibitors (AG490, JI, EGCG, fludarabine) on the Interleukin-6 (IL-6) production in the LPS-stimulated RAW264.7 murine macrophages.

(A) Cells (1.5 x 10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 h and then IL-6 production was determined from the culture supernatants of cells stimulated with LPS (1 μg/mL) in the presence of NF-κB inhibitors (PDCT, TPCK, parthenolide) for 24 h (6.25 to 50 μM). IL-6 production was determined by ELISA. The measurement of IL-6 was performed in duplicate. Error bars indicate ± S.D. *, P<0.05, **, P<0.01, ***, P<0.001, significant when compared with LPS positive control. (B) Cells (1.5 x 10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 h and then IL-6 production was determined from the culture supernatants of cells stimulated with LPS (1 μg/mL) in the presence of MAPK inhibitors (SP, PD, SB 203580) for 24 h (5 to 20 μM). IL-6 production was determined by ELISA. The measurement of IL-6 was performed in duplicate. Error bars

indicate ± S.D. *, $P<0.05$, **, $P<0.01$, significant when compared with LPS positive control. (C) Cells (1.5 x 10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 h and then IL-6 production was determined from the culture supernatants of cells stimulated with LPS (1 μg/mL) in the presence of STAT inhibitors (AG490, JI, EGCG, fludarabine) for 24 h (5 to 20 μM). IL-6 production was determined by ELISA. The measurement of IL-6 was performed in triplicate. Error bars indicate ± S.D. **, $P<0.01$, significant when compared with LPS positive control.

4.2.3. Effect of DPHC on the phosphorylation of MAPKs in LPS-stimulated RAW 264.7 cells

The macrophage activates the MAPK signaling pathways (i.e., p38, JNK, ERK) by LPS stimulation (Meng, Lowell 1997, Qi, Shelhamer 2005). MAPK consists of p38 kinases, extracellular signal-regulated protein kinases (ERKs), and c-Jun N-terminal kinases/stress-activated protein kinases; in addition, it is related to the induction and progression of diseases including cancer, autoimmune diseases, and diabetes (Seger, Krebs 1995, Plotnikov et al. 2011). Therefore, we examined the effect of DPHC on the LPS-induced activity of MAPKs at each time course (0, 5, 15, 30, 60, 120, 240, and 360 min) by western blot. When the RAW 264.7 cells stimulated LPS (1 μg/mL) at each time course (0, 5, 15, 30, 60, 120, 240, and 360 min), the phosphorylation of MAPKs (p38, JNK, and ERK) increased at 15 min after LPS stimulation (data not shown). We then investigated whether or not DPHC affects the down-regulation of the MAPK signal pathway using western blot. DPHC (12.5 and 100 μM) had no effect on the LPS-stimulation activation of MAPKs (Fig. 13). These results suggest that DPHC does not affect the MAPK pathways.

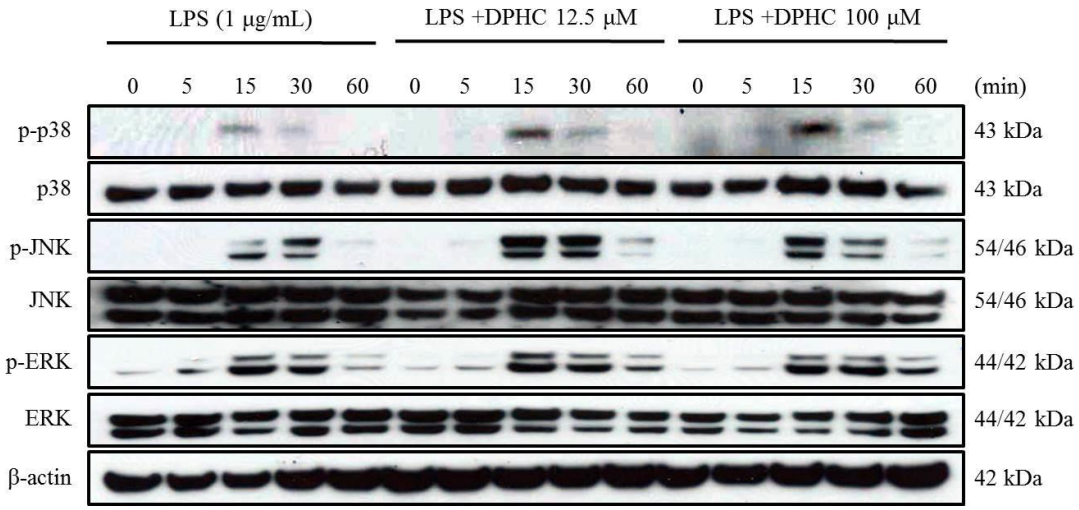

Figure 13. Effect of diphlorethohydroxycarmalol (DPHC) on expression of MAPKs phosphorylation in LPS-stimulated RAW264.7 macrophages.

RAW264.7 cells (7.5×10^5 cells/mL) were pre-incubated for 18 h. And cell were stimulated with LPS (1 μg/mL) and treated presence or absence of DPHC (12.5, 100 μM) for indicated time. Expression of MAPKs phosphorylation was determined by Western blotting of whole cell lysates with the indicated antibodies.

4.2.4. Effect of DPHC on the phosphorylation of STATs in LPS-stimulated RAW 264.7 cells

The activation of STATs, such as LPS-stimulated type-Ⅰ interferon activation (Bode, Ehlting & Haussinger 2012, Yamaoka et al. 1998, Ehlting et al. 2011, Rhee et al. 2003, Ohmori, Hamilton 2001a), is also crucial to inflammatory response. Therefore, we measured the LPS-induced phosphorylation of STATs (STAT1, STAT3, and STAT5; data not shown) and determined the effect of DPHC on such phosphorylation. Samavati et al (2009) stimulated cells with LPS at each time course (0, 5, 15, and 30 min and 1, 2, 3, 4, and 6 h) and found that STAT1 and STAT3 were phosphorylated at 2 h. Hu et al (2012) stimulated cells with LPS at each time course (0 and 30 min and 1, 2, 3, and 4 h) and found that STAT5 was phosphorylated by LPS at 2 h. Thus, we investigated whether or not DPHC affect the down-regulation of the STAT signaling pathway by western blot. RAW 264.7 cells were treated with LPS or LPS (1 μg/mL) and DPHC (12.5 and 100 μM) for 120, 240, and 360 min. As shown in Fig. 14, DPHC weakly reduced STAT5 phosphorylation and total STAT5, and DPHC (12.5 and 100 μM) had no effect on the LPS-stimulated activation of STATs (STAT1, STAT3). These results suggest that DPHC weakly affect STAT5 pathway, and does not affect the STAT1 and STAT3 pathways .

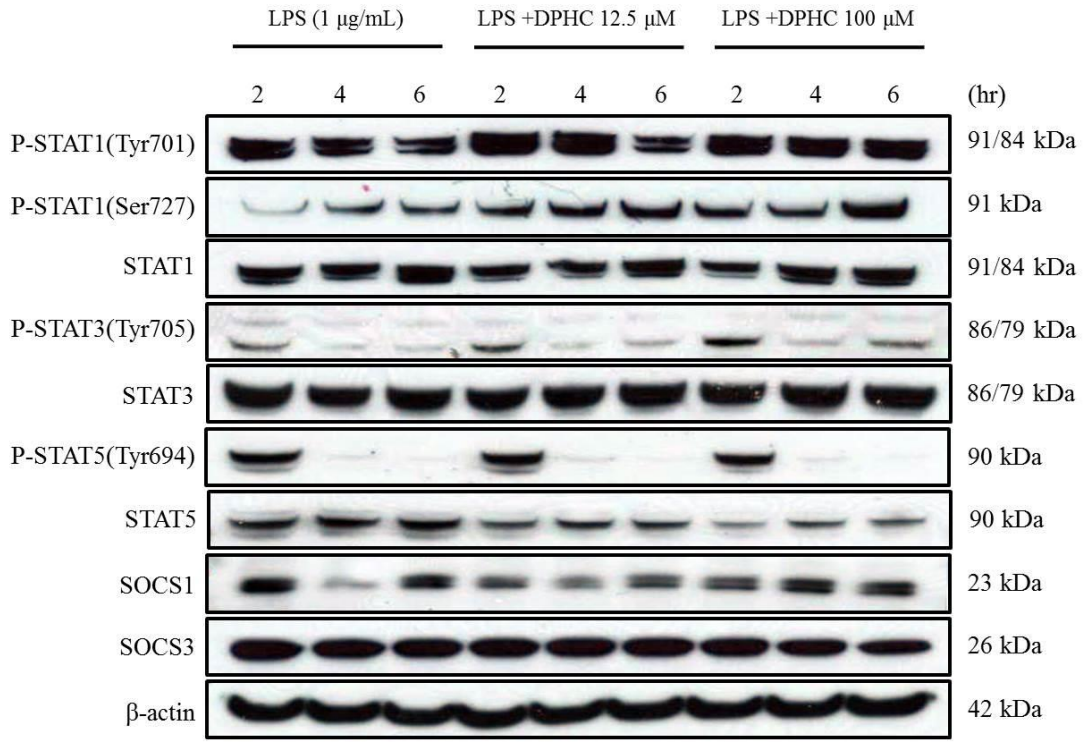

Figure 14. Effect of diphlorethohydroxycarmalol (DPHC) on expression of STAT1 (Tyr701, Ser727), STAT3, STAT5 (Tyr694) phosphorylation in LPS-stimulated RAW264.7 macrophages.

RAW264.7 cells (7.5×10^5 cells/mL) were pre-incubated for 18 h. And cell were stimulated with LPS (1 μg/mL) and treated presence or absence of DPHC (12.5, 100 μM) for indicated time. The phosphorylation of STAT1 (Tyr701, Ser727), STAT3, STAT5 (Tyr694) was determined by Western blotting of whole cell lysates with the indicated antibodies.

4.2.5. Effect of DPHC on the phosphorylation of NF-κB in LPS-stimulated RAW 264.7 cells

NF-κB is basic transcription factor and a central inflammatory mediator involved in the genetic induction of cellular proliferation, inflammatory cytokines, and pathogen-derived substances (Akira, Takeda 2004, Park et al. 2009, Kagan, Medzhitov 2006, Lu, Yeh & Ohashi 2008, Kawai, Akira 2006, Li, Verma 2002). When RAW 264.7cells were stimulated with LPS (1 μg/mL) at each time course (0, 5, 15, 30, 60, 120, 240, and 360 min), the activation of NF- κB (p65, IκB-α) significantly increased at 5, 60, and 240 min after LPS stimulation (data not shown). Thus, we investigated whether or not DPHC inhibits the phosphorylation of NF-κB at each time course (0, 5, 15, 30, 60, 120, 240, and 360 min) by western blot. DPHC (12.5 and 100 μM) inhibited the LPS-induced phosphorylation of NF-κB at 5, 15, 30, and 60 min and 6 h (Fig. 15).

We examined the activity of NF-κB (phospho-p65, p50) by confocal laser scanning microscopy to confirm whether or not DPHC affects nuclear translocation. DPHC inhibited the LPS-induced translocation of NF-κB (phosphor-p65, p50) at 30, 60, and 360 min (Figs. 16 and 17). These results suggest that DPHC inhibits IL-6 production via the NF-κB signaling pathway.

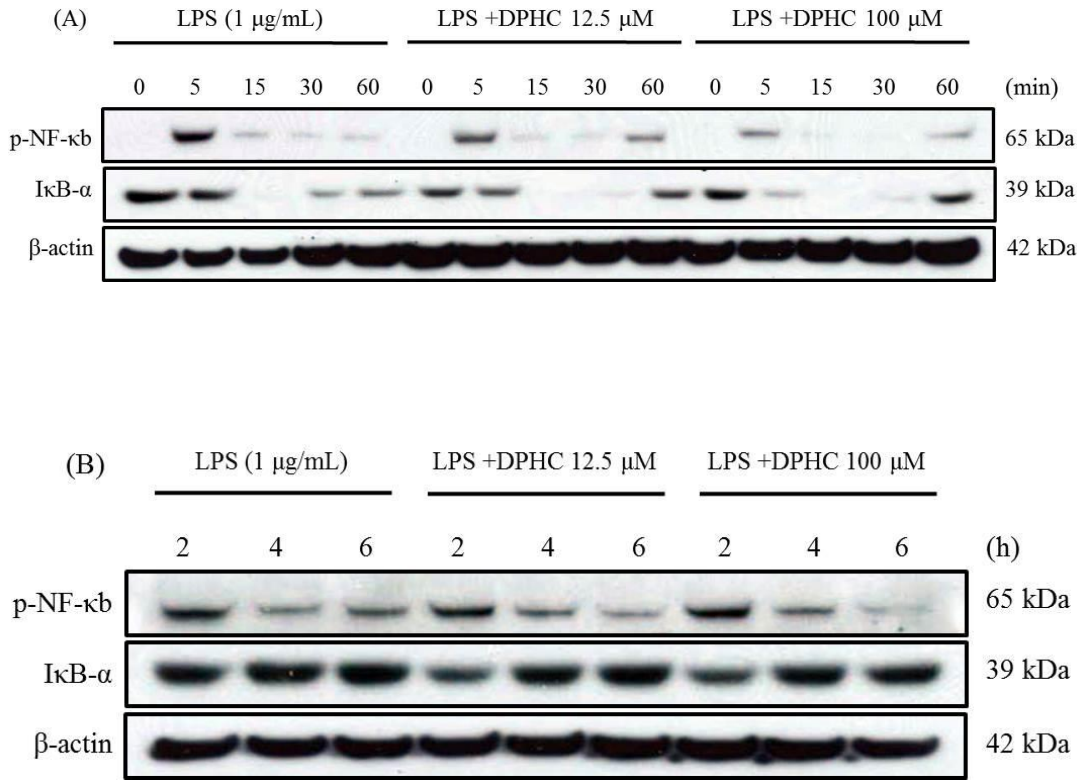

Figure 15. Effect of DPHC on the NF-κB phosphorylation in LPS-stimulated RAW264.7 macrophages.

(A) RAW264.7 cells (7.5×10^5 cells/mL) were pre-incubated for 18 h. And cell were stimulated with LPS (1 μg/mL) and treated presence or absence of DPHC (12.5, 100 μM) for indicated time. Expression of NF-κB phosphorylation was determined by Western blotting of whole cell lysates with the indicated antibodies. (B) RAW264.7 cells (7.5×10^5 cells/mL) were pre-incubated for 18 h. And cell were stimulated with LPS (1 μg/mL) and treated presence or absence of DPHC (12.5, 100 μM) for 2, 4, 6 h. Expression of NF-κB phosphorylation was determined by Western blotting of whole cell lysates with the indicated antibodies.

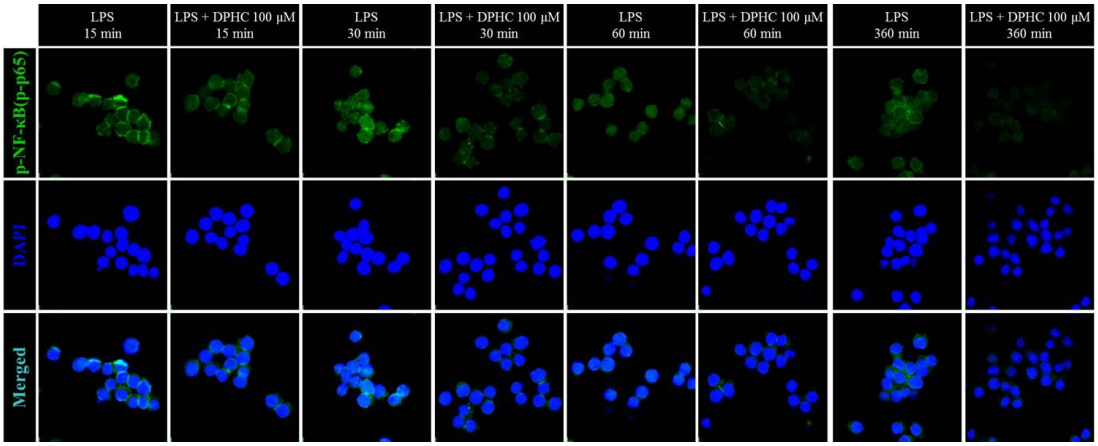

Figure 16. Effect of DPHC on the NF-κB phosphorylation in LPS-stimulated RAW264.7 macrophages.

Cells (2.0 x 10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 h. Then cells were stimulated with LPS (1 μg/mL) and treated presence or absence of DPHC (100 μM) for indicated time. Immunofluorescence stain of phospho-NF-κB was stained with DyLight488-conjugated 2^{nd} antibody and the fluorescence was identified using confocal microscopy (FV500, OLYMPUS) and the images were acquired at constant PMT, gain, offset, magnification (40x oil immersion objective with zoom factor of 3.0) and resolution. These data are representative of three independent experiments.

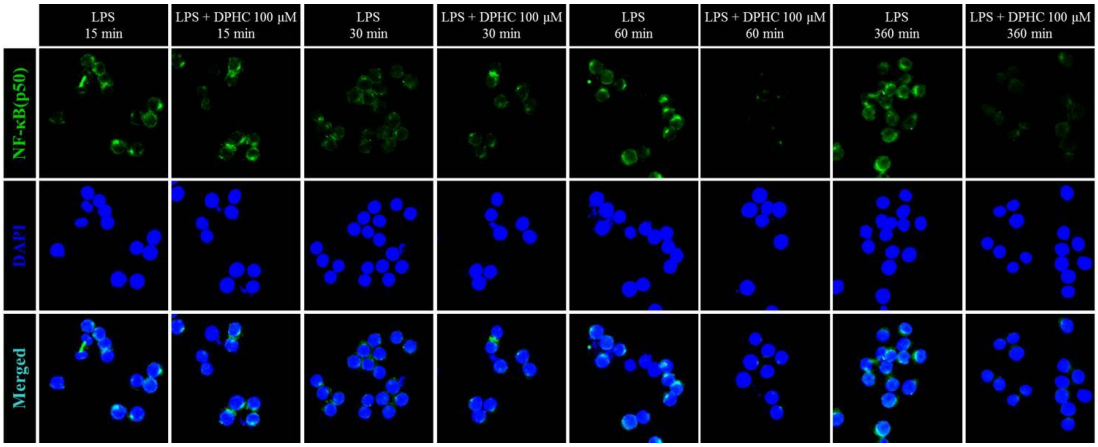

Figure 17. Effect of DPHC on the NF-κB phosphorylation in LPS-stimulated RAW264.7 macrophages.

Cells (2.0 x 10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 h. Then cells were stimulated with LPS (1 μg/mL) and treated presence or absence of DPHC (100 μM) for indicated time. Immunofluorescence stain of total-NF-κB (p50) was stained with DyLight488-conjugated 2^{nd} antibody and the fluorescence was identified using confocal microscopy (FV500, OLYMPUS) and the images were acquired at constant PMT, gain, offset, magnification (40x oil immersion objective with zoom factor of 3.0) and resolution. These data are representative of three independent experiments.

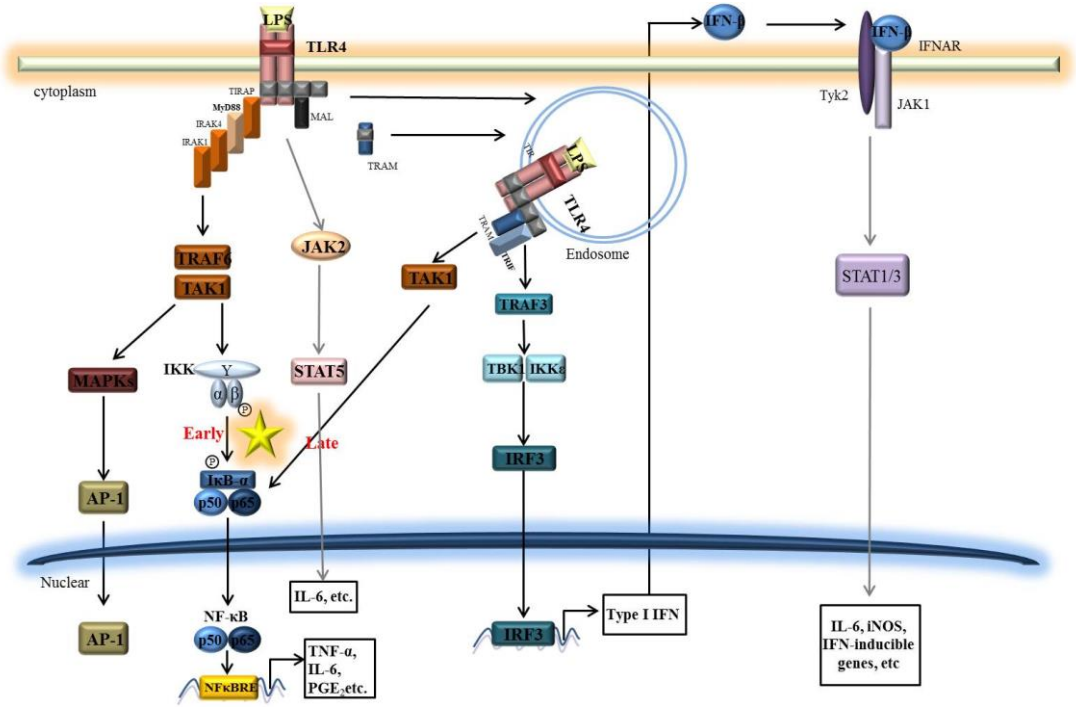

Figure 18. Inhibitory effect and action mechanism of diphlorethohydroxycarmalol (DPHC) on the production of interleukin-6 in lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells.

5. Discussion

In the present study, we investigated the effect of DPHC, isolated from the brown alga *ishige okamurae* on the production of inflammatory mediators, such as IL-6, TNF-α, NO, and PGE_2, in LPS-stimulated murine macrophage RAW 264.7 cells. We also investigated the action mechanism of DPHC in these cells. We found that DPHC inhibits the production of IL-6 via the NF-κB signaling pathway in these cells.

DPHC isolated from *I.okamurae* protects against radiation-induced cell damage and high glucose-induced-oxidative stress (Heo et al. 2010, Heo et al. 2009). Moreover, DPHC increases prostaglandin E_2 via the expression of COX-1 and COX-2 and attenuates UVB-induced cell damage by absorbing UVB rays and enhancing the antioxidant system in human HaCaT keratinocytes (Kang et al. 2012, Piao et al. 2013).

Inflammation is a complex of interaction with cells and factors that can arise in various tissues in response to autoimmune injury and infection. Inflammation presents in various diseases, such as stress, metabolic disorders, and obesity (Nathan 2002, Wellen, Hotamisligil 2005, Hotamisligil 2006). Various cytokines participate in inflammation (Hanada, Yoshimura 2002). For example, IL-6 mediates the hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin in cultures of human and mouse liver cells (Nemeth et al. 2004). IL-6 is also target in molecular therapy because inhibition of IL-6 treats inflammatory diseases related to it, including rheumatoid arthritis, Castleman's disease, juvenile idiopathic arthritis, Crohn's disease, and other chronic inflammatory diseases (Nishimoto, Kishimoto 2004, Gabay 2006).

IL-6 regulation is especially important in regulating the acute-phase response in infection and injury (Heinrich et al. 2003). More specifically, the level of IL-6 is high in the plasma of

septic patients (Hack et al. 1989). Hohki and Ohguro et al. reported that IL-6 blockade is inhibited by the suppresses of inflammatory T-helper17 responses mediated via mechanisms from TNF blockade in autoimmune uveoretinitis; thus, IL-6 blockade may have a therapeutic effect on human ocular inflammation (Hohki et al. 2010). Therefore, we investigated whether or not DPHC inhibits IL-6 production in LPS-stimulated RAW 264.7 cells. As shown in Fig. 10, DPHC (25, 50, and 100 μM) strongly inhibited IL-6 production in LPS-induced RAW 264.7 cells in a dose-dependent manner (Fig. 10). In addition to IL-6 cytokines, TNF-α also is a crucial cytokine in inflammation and a central regulator of inflammation. TNF inhibition effectively treats rheumatoid arthritis and inflammatory disease (Esposito, Cuzzocrea 2009, Feldmann, Maini 2001). We also examined the effect of DPHC on TNF-α production in LPS-stimulated RAW 264.7 cells, but the results suggest that DPHC does not affect TNF-α production (Fig. 9). NO is an important biological mediator that is gaining wide recognition, and NO production and nitric oxide synthase (NOS) expression are important to the pathogenesis of various diseases, such as inflammation and immune disorders (Nussler, Billiar 1993). Human NOS is observed in monocytes or macrophages in patients with infectious or inflammatory disease (MacMicking, Xie & Nathan 1997). iNOS gene expression is controlled by NF-κB, JAK-STAT, activator protein-1 (AP-1) and signaling pathway. Prostaglandins are small molecular derivatives of arachidonic acid, and PGE_2 is an essential homeostatic factor and a key mediator of immunophathology that supports local inflammation. Prostaglandins produce COX-2 (Kalinski 2012, Phipps, Stein & Roper 1991), which can be regulated rapidly and transiently by pro-inflammatory mediators and stimulators, such as cytokines, endotoxins, and growth factors (Surh et al. 2001). COX-2 is also controlled by the MAPK signaling pathway, which activates the transcription factors of COX-2, including AP-1 and NF-κB (Surh et al. 2001, Kleinert et al. 1998, Samardzic et al. 2001, Fukata, Abreu 2008, Wu, Meydani 2004, Subbaramaiah et al. 2000, Subbaramaiah, Dannenberg 2003). NO and PGE_2 are factors related to inflammatory response; thus, NO, iNOS, PGE_2, and COX-2 are potential molecular targets for pre-

inflammatory responses (Kang et al. 2013, Brooke et al. 2013). In this study, we confirmed that DPHC (12.5, 25, 50, and 100 μM) weakly inhibited NO and PGE_2 production, and inflammatory factors, by LPS-induced RAW 264.7 cells (Figs. 7 and 8). DPHC (25, 50, and 100 μM) also weakly inhibited iNOS and COX-2 expression by LPS-induced RAW 264.7 cells (Fig. 11). Therefore, we focused on the inhibitory effect and action mechanism of DPHC on the production of IL-6 in LPS-stimulated murine macrophage RAW 264.7 cells.

We were examined the inhibitory mechanism of IL-6 production by DPHC in LPS-induced RAW 264.7 cells. LPS-induced IL-6 gene expression is mediated by various signaling pathways, including MAPK, STATs, and NF-κB (Sweet, Hume 1996, Olsnes, Olofsson & Aarstad 2011, Minogue, Barrett & Lynch 2012). Lycopene inhibits LPS-induced IL-6 production by suppressing the activation of ERK, p38, and NF-κB but has no effect on TNF-α. Theaflavin suppresses LPS-induced IL-6, monocyte chemoattractant protein-1, and intercellular adhesion molecule-1 production via the blockade of the NF-κB and MAPK signaling pathways in bone-marrow-derived macrophages isolated from ICR mice (Kim, Joo 2011, Feng, Ling & Duan 2010). We evaluated the effect of DPHC on the phosphorylation of MAPKs and STATs. Although several compounds or extracts inhibit IL-6 production via the MAPK and STATs signaling pathways, DPHC did not have any effect on MAPK and STATs phosphorylation in our experiments (Figs. 13 and 14). The cytokine-activated JAK-signaling transducer and activator of transcription (STAT) pathway considerably affect the immune system. The JAK-STAT signaling pathway regulates all cell type involved in initiation, propagation, and inflammation (Shuai, Liu 2003, O'Shea, Murray 2008). IL-6 is also required in the up-regulation of iNOS and COX-2 through the JAK-STAT signal pathway (O'Shea, Murray 2008, Kalinski 2012, Dawn et al. 2004). IL-6 production regulates the JAK-STAT mechanism, and STAT3 tyrosine phosphorylation is critical for IL-6 production in response to LPS and live bacteria (Samavati et al. 2009, Kimura et al. 2005). Thus, we examined whether or not STAT inhibitors (AG490, JI, EGCG, and fluoroadenine) inhibit IL-

6 cytokines. The results suggest that IL-6 production is related to STATs (Fig. 12). DPHC weakly inhibited iNOS protein in LPS-stimulated RAW 264.7 cells (Fig. 11). Although several reports determined that compound or extracts inhibited IL-6 production via MAPK, STATs signaling pathway, our findings showed that DPHC did not have any effect on MAPK, STATs phosphorylation in our experiments (Fig. 12 ~ 14).

We confirmed the effect of DPHC on the NF-κB signaling pathway, which activates IL-6 production by LPS. NF-κB is one of the basic transcription factors involved in the gene induction of cellular proliferation, inflammatory cytokines, and pathogen-derived substances (Li, Verma 2002).The products of NF-κB-driven genes, including IL-6, have been a major breakthrough in rheumatoid arthritis patients (NF-kB and its relevance to arthritis and inflammation). We tested the effect of DPHC on NF-κB phosphorylation and on nuclear translocation of p65 and p50 subunits in LPS-stimulated RAW 264.7 cells by western blot and confocal scanning microscopy to examine the inhibitory mechanism of IL-6 production for DPHC (Fig. 4). DPHC (12.5 and 100 μM) inhibited NF-κB phosphorylation at 5, 60, and 360 min, compared with that in the positive control group. DPHC also suppressed LPS-stimulated I-kappaB (IκB) degradation at 60 and 360 min but weakly inhibited it at 5 and 30 min; NF-κB phosphorylation was inhibited at 5, 15, 30, and 60, and 360 min (Fig. 15). DPHC inhibited the nuclear translocation of phosphor-p65 and p50 proteins (Figs. 16 and 17). The NF-κB p50 subunit may function as the promoter region of IL-6 with IκB-zeta (Yamamoto et al. 2004). Thus, our finding that DPHC inhibited p50 proteins by confocal scanning microscopy is similar (Fig. 17). We also found that NF-κB inhibitors (PDTC, TPCK, and parthenolide) and MAPK inhibitors (SP600125, PD098059, and SB203580), inhibited IL-6 cytokines (Fig. 12). However, DPHC strongly inhibited the NF-κB, and not the MAPK signal pathway (Figs. 13 and 15~17). These results suggest that the inhibitory effect of DPHC on IL-6 cytokine is exerted through the down-regulation of the NF-κB mechanism rather than the STAT or MAPK mechanism. Thus, the STAT mechanism is also

related to IL-6 production.

In conclusion, DPHC modulates inflammatory conditions in murine macrophage RAW 264.7 cells and strongly suppresses the production of IL-6 and weakly affect that of other inflammatory mediators. DPHC also strongly suppresses the NF-κB pathway and weakly affect STAT5 pathway. Thus, DPHC suppresses the production of IL-6 via the down-regulation of the NF-κB pathway in LPS-stimulated RAW 264.7 cells. These findings enhance the importance of DPHC as anti-inflammatory material in the treatment of inflammatory diseases (Fig. 18).

6. References

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감사의 글

제가 뜻하는 바 가고자 하는 길을 위해 지나온 2년간의 석사과정이 어느덧 막바지에 이르렀습니다. 새로운 길을 선택한 만큼 여러 가지 많은 생각과 더불어 두려움과 설렘이 공존하던 시간이었습니다. 돌아보면 그 시간은 많은 실수와 아쉬움, 부족함 속에서도 한 단계 더 성장할 수 있는 값진 시간이었으며, 이 경험을 발판 삼아 앞으로 다가올 새로운 도전의 길에 힘차게 도약해 보려 합니다. 지난 2년간 많은 분들의 격려와 도움이 있었기에 석사과정을 잘 마무리 할 수 있었습니다. 이 지면을 빌어 물심양면으로 저를 아껴주신 많은 분들께 감사의 마음을 전하고자 합니다.

전공과 실험에 대해 아무 것도 모르는 백지상태에서 시작해야 했던 저를 끝까지 변함없이 믿고 지도해주신 유은숙 교수님께 깊은 감사의 말씀을 드립니다. 새로운 분야의 공부와 실험을 시작하면서 새로움과 궁금증으로 인해 그만큼 부족함도 많았지만 교수님의 지도 아래 넓은 안목을 가지고 다양한 실험과 폭넓은 공부를 할 수 있어 행복했습니다. 논문의 부족한 부분에 대해 다양한 측면에서 조언을 아끼지 않으신 강희경 교수님과 박덕배 교수님께도 진심을 담아 감사의 마음을 전합니다. 또한 늘 따듯한 미소와 관심으로 지켜봐 주신 은수용 교수님, 조문제 교수님, 현진원 교수님, 고영상 교수님, 정성철 교수님, 이영기 교수님, 강현욱 교수님, 정영배 교수님, 이근화 교수님, 김수영 교수님, 윤상필 교수님께 깊은 감사의 말씀을 드립니다.

그간 약리학 교실에서 함께 생활하며 저에게 많은 것을 가르쳐주셨던 선배님들께 감사의 마음을 전합니다. 저에게 처음으로 다양한 실험을 가르쳐 주시고 기초부터 그 이상의 많은 것을 배울 수 있게 도와주신 강경진 박사님과 항상 웃으며 즐거운 마음으로 보다 폭넓은 시야를 가지고 연구할 수 있도록 많은 것을 가르쳐 주신 한상철 선배님, 항상 힘을 주며 선배로써 조언을 아끼지 않는 강정일 박사님, 저의 사소한 고민부터 실험까지 항상 옆에서 챙겨주고 같이 고민해주던 은아 언니께 감사의 마음을 전합니다. 그리고 실험실에서 아무것도 모르는 저에게 많은 의지가 되어주었던 동기 동환이와 친구 은지, 또한

약리학교실에 처음 들어왔을 때 석 달 동안 많은 힘이 되어주었던 유나와 늘 언니처럼 챙겨주고 힘이 되어준 후배 수연이, 짧은 기간이었지만 함께 의지했던 문정이에게 감사의 마음을 전합니다. 생리학 교실에 항상 밝은 모습으로 맞아주시는 양윤실 선생님, 오금희 선생님, 박지연 선생님, 항상 많은 조언과 격려를 아끼지 않는 생화학 교실에 김영미 선생님, 김기천 선생님, 손지현 선생님, 서가영 선생님, 지금은 실험실에 없지만 있는 동안 저에게 많은 힘을 주었던 저의 동기이자 친구인 유재에게도 감사의 마음을 전합니다.

바쁘다는 핑계로 자주 만나지 못하고, 연락도 못해 늘 미안한 친구, 항상 먼저 연락해주고 챙겨주는 나의 안식처와 같은 친구들 정윤이, 유리, 아름이, 은정이, 수희, 희진이, 진주, 선미, 지혜, 주현이, 온유, 미숙이, 재훈이, 승진이, 성욱이, 유철이, 관효에게 감사의 마음을 전합니다. 그리고 서울에 있어서 자주 만나지 못하고, 연락도 자주 못하지만 언제 만나든, 언제 연락하든 항상 편안한 내 친구 은비와 동생 유진이, 또한 너무 귀여운 동생들 윤영이, 유라, 은지(고), 유나, 혜란이, 혜연이, 늘 아껴주고 예뻐 해주는 근영언니, 해선언니, 지운이, 재윤이, 그리고 후배를 늘 먼저 생각해주고 챙겨주는 미소언니, 진범오빠, 영문오빠, 정문오빠, 현웅오빠, 동현오빠, 만날 때 마다 기분 좋은 지선언니, 수미, 영미, 지혜(강), 지혜(김), 은지에게도 고맙다는 말을 전하고 싶습니다.

사랑하는 가족에게도 감사의 마음을 전합니다. 항상 손녀를 사랑스럽게 봐주시는 성할머니와 외할머니, 작은 딸이 하는 모든 일을 항상 믿고 지켜봐 주시면서 응원해주시는 우리 아버지와 어머니, 부족한 며느리 항상 예쁘게 봐주시고 학업에 열중 할 수 있도록 용기를 주신 시아버지와 시어머니, 부족한 동생이지만 늘 사랑으로 감싸주며 지원을 아끼지 않는 우리 희진 언니, 작은 누나 묵묵하게 지켜 봐주는 듬직한 우리 남동생 동원이, 힘과 용기를 아낌없이 주는 우리 신영언니, 모두들 사랑하고 감사합니다. 마지막으로 누구도 대신할 수 없는 나의 활력소이자 든든한 지원군인 내 남편 박철형 오빠! 오빠가 보여준 용기와 믿음과 사랑이 있었기에 여기까지 올 수 있었습니다. 늘 나를 행복하게 해주는 내 남편에게 사랑과 감사의 마음을 전합니다.

도와주신 모든 분들의 행복과 건승을 빌겠습니다. 고맙습니다.