A THESIS
FOR THE DEGREE OF MASTER OF ENGINEERING

Anti-inflammatory Activity of Sulforaphene

Kyeong-Yeol Park

Department of Food Science and Engineering
GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

2014. 2.

Anti-inflammatory Activity of Sulforaphene

Kyeong-Yeol Park
(Supervised by professor Sang-Bin Lim)

A thesis submitted in partial fulfillment of the requirement
for the degree of Master of Engineering

2014. 02.

This thesis has been examined and approved.

Jin-Hwan Ha, Thesis director, Prof. of Food Science and Engineering

Hyun Jung Kim, Prof. of Food Science and Engineering

Sang-Bin Lim, Prof. of Food Science and Engineering

February 2014

Department of Food Science and Engineering
GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

Contents

요약 ····· iii

List of Figures ····· iv

SUMMARY ····· v

1. INTRODUCTION ····· 1

2. MATERIALS AND METHODS ····· 5

2.1 Chemicals ····· 5

2.2 Cell culture ····· 6

2.3 Cell viability assay ····· 6

2.4 NO production assay ····· 6

2.5 PGE_2 production assay ····· 7

2.6 Pro-inflammatory cytokines production assay ····· 7

2.7 Immunoblot analysis ····· 7

2.8 Total cellular RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) assay ····· 8

2.9 Transient transfection and luciferase assay ····· 9

2.10 Statistical analysis ····· 9

3. RESULTS AND DISCUSSION ····· 11
3.1 Cytotoxicity of sulforaphene ····· 11
3.2 Inhibitory effect of sulforaphene on LPS-stimulated NO production ····· 13
3.3 Inhibitory effect of sulforaphene on LPS-stimulated PGE_2 production ····· 15
3.4 Inhibitory effect of sulforaphene on LPS-stimulated pro-inflammatory cytokines production ····· 17
3.5 Inhibitory effect of sulforaphene on iNOS and COX-2 protein expression ····· 20
3.6 Inhibitory effect of sulforaphene on mRNA expression ····· 22
3.7 Inhibitory effect of sulforaphene on NF-κB transcriptional activity ····· 25
3.8 Inhibitory effect of sulforaphene on degradation of Iκb-α and activation of NF-κB ····· 27

REFERENCES ····· 31

요약

무에 함유되어 있는 isothiocyanates류인 sulforaphene의 항염증 활성에 관한 연구가 아직 없는 실정이다. 따라서 RAW 264.7 세포주를 LPS로 자극하여 sulforaphene의 iNOS, COX-2, 전염증성 cytokines와 같은 염증 유발 인자에 대한 저해활성을 측정하였다. sulforaphene은 농도 의존적으로 LPS 자극에 의해 생성된 NO의 양을 감소시켰다. 이와 마찬가지로 NO를 생성하는 iNOS 단백질과 iNOS mRNA도 sulforaphene에 의해 감소되는 것을 확인하였다. 또한 전염증성 cytokine인 IL-6와 IL-6 mRNA도 sulforaphene에 의해 농도 의존적으로 저해되었다. 이러한 sulforaphene의 저해능력은 LPS 자극에 의한 Iκb-a 의 분해와 NF-κB의 활성화를 저해함으로써 전염증성 매개인자들에 대한 억제를 나타내는 것으로 사료된다. 따라서 본 연구결과는 NO에 의한 염증 질환과 IL-6에 의한 류마티스 관절염 혹은 동맥경화증 치료제로서의 sulforaphene의 가능성을 제시한다.

List of Figures

Fig. 1. The chemical structure of sulforaphene (4-methylsulfinyl-3-butenyl isothiocyanate)

Fig. 2. Cell viability of sulforaphene in LPS-stimulated RAW 264.7 cells

Fig. 3. Inhibitory effects of sulforaphene on NO production in LPS-stimulated RAW 264.7 cells

Fig. 4. Inhibitory effects of sulforaphene on PGE_2 production in LPS-stimulated RAW 264.7 cells

Fig. 5. Inhibitory effects of sulforaphene on TNF-α (A), IL-1β (B), and IL-6 (C) production in LPS-stimulated RAW 264.7 cells

Fig. 6. Inhibitory effects of sulforaphene on iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells

Fig. 7. Inhibitory effects of sulforaphene on mRNA expression in LPS-stimulated RAW 264.7 cells

Fig. 8. Inhibitory effects of sulforaphene on luciferase activity in LPS-stimulated RAW 264.7 cells

Fig. 9. Inhibitory effects of sulforaphene on degradation of Iκb-α depending on exposure time of LPS in RAW 264.7 cells

Fig. 10. Inhibitory effects of sulforaphene on degradation of Iκb-α and activation of NF-κB in LPS-stimulated RAW 264.7 cells

ABSTRACT

The inhibitory activities against the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines dependent on regulation of nuclear factor-kappa B(NF-κB) in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells using sulforaphene (0~10 μM) were investigated. Sulforaphene significantly reduced NO production induced by LPS in a concentration-dependent manner, and iNOS protein and mRNA caused NO production was down-regulated by sulforaphene. Interleukin (IL)-6 production and mRNA as pro-inflammatory mediator were suppressed in a concentration-dependent manner by sulforaphene. However, prostaglandin E_2 (PGE_2) and pro-inflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1β was not inhibited by sulforaphene. Sulforaphene reduced NF-κB activity and inhibited LPS-mediated inhibitory kappa b (Iκb)-α degradation in cytoplasm and translocation of NF-κB into nucleus within 20 min. These results suggest that sulforaphene could suppress inflammatory responses through the inhibition of iNOS and IL-6 pathway by regulating NF-κB in LPS-stimulated macrophages.

1. INTRODUCTION

Radishes (*Raphanus sativus* L.) are the plant family of cruciferae, and roots and leaves of radish have been globally used to treat cancer and other diseases (1). Many studies on radish have demonstrated biological activities including antimicrobial (2), antioxidant (3), antimutor (4), and antiproliferative activities (5, 6). Those activities appear because radishes contain many bioactive phytochemicals such as phenolic compounds, glucosinolates, and various useful enzymes (1). Especially isothiocyanates being converted from glucosinolates by endogenous myrosinase had anticancer activity (7).

Sulforaphene (4-methylsulfinyl-3-butenyl isothiocyanate, shown in Fig. 1), a kind of isothiocyanates derived from glucoraphenin rich in radish, has been known to increase Bax and decrease Bcl2 protein expression associated with apoptosis in Lovo, HCT-116, and HT-29 human colon cancer cell lines (6, 8). It also has been shown antiproliferative effects in human and murine erythroleukemic cells (K562 and FL), human T-lymphoid cells (Jurkat and H9), and human cervix carcinoma cells (HeLa and H3-T1-1) (9, 10). Moreover, sulforaphene showed 1.3-1.5 times more potent activity than sulforaphane in vitro antimutagenic activity in the TA100 strain of *Salmonella typhimurium* (11).

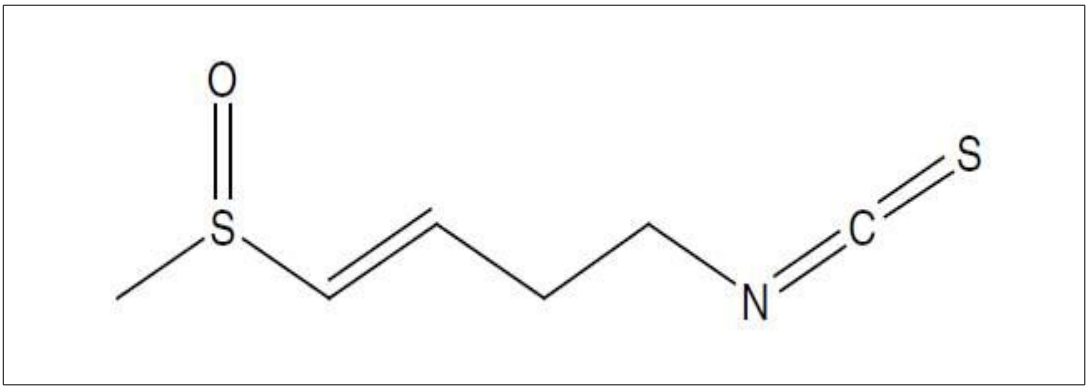


Fig. 1. The chemical structure of sulforaphene (4-methylsulfinyl-3-butenyl isothiocyanate)

Inflammation is a major defense mechanism of the immune system against an invading pathogen or harmful injury, but is known to be the cause of many diseases (12-14). The intensity of inflammation response is important in immune response. Immunodeficiency resulting from deficient inflammation responses can lead to infection and cancer. In contrast, excessive inflammation responses bring about diseases such as rheumatoid arthritis, atherosclerosis, Crohn's disease, diabetes, and Alzheimer's disease (15).

Lipopolysaccharide (LPS) is a major constituent of gram-negative bacteria, acts as endotoxins, and can induce activation of macrophage (16). The macrophage is stimulated by LPS, which produces excessive inflammation product such as nitric oxide (NO), prostaglandin E_2 (PGE_2), and pro-inflammatory cytokines (17-19).

NO is a free radical and is synthesized from L-arginine using molecular oxygen and NADPH by NO synthases (NOSs) enzymes (20). iNOS among NOSs is affected by simulation of LPS, which generates large amount of NO. NO plays a variety of roles in host defense against tumor cells, biological mediator as neurotransmitters, control of blood pressure, and vasodilatation.

However, an excess level NO in cell can cause oxidative damage and inflammatory disorders by generating reactive radical such as peroxynitrite (21).

Prostaglandins (PGs) are generated by conversion of arachidonic acid by cyclooxygenase (COX) enzymes. When inflammatory response occurs, COX-2 is expressed, and PGE_2 is produced by COX-2. PGE_2 is an inflammatory product and has been reported to create cancers of colon, lung, and breast (22-24).

Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and interleukin (IL)-6 plays an important role in inflammation responses (25-27). Especially, IL-6 has been implicated in many diseases including rheumatoid arthritis, atherosclerosis, multiple myeloma, AIDS, sepsis and keloid (28).

Nuclear factor-kappa B (NF-κB) as a transcription factor remains inactivated state that has bound with inhibitory kappa B-α (IκB-α) in the cytoplasm of cells. When Inhibitory kappa B kinase (IKK) acts on complex of NF-κB and IκB-α, IKK can induce phosphorylation, ubiquitination, and degradation of Iκ B-α in order. NF-κB is activated and translocated to the nucleus, and binds to relevant DNA binding site on the promoter of inflammatory genes such as iNOS, COX-2, TNF-α and ILs (29).

Recent study reported that sulforaphene inhibited COX-2 expression in bladder cancer cells (30), but anti-inflammation mechanism of sulforaphene in macrophage related to NF-κB as an important key of inflammation has not been fully understood yet. Thus we investigated the inhibition effects on the expression of iNOS, COX-2, and pro-inflammatory cytokines dependent on

regulation of NF-κB in LPS-stimulated RAW 264.7 cells by treating sulforaphene.

2. MATERIALS AND METHODS

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). LPS (*Escherichia coli* 0111:B4) was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Anti-iNOS antibody was purchased from Calbiochem (San Diego, CA, USA). Anti-COX-2 antibody was acquired from BD Transduction Laboratories (Lexington, KY, USA). Other primary antibodies, secondary antibodies and sulforaphene were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) was obtained from GE Healthcare (Buckinghamshire, UK). The PCR primers were purchased from Bioneer (Seoul, Korea).

2.2. Cell culture

RAW 264.7 murine macrophage cell line was purchased from Korean Cell Line Bank (Seoul, Korea). The cells were cultured in DMEM medium containing 100 unit/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated FBS, and maintained at 37℃ in a humidified atmosphere of 5% CO_2.

2.3. Cell viability assay

Cell viability was determined by WST-1 kit (Daeil Lab Service, Seoul, Korea). RAW 264.7 cells were cultured in 96-well plates (2×10^5 cells/well) for 24 hr, followed by the treatment with LPS (1 μg/mL) in the presence of

different concentrations (0~20 μM) of sulforaphene. After a 24 hr incubation, 5 μL of WST-1 solution was added to the medium and maintained at 37℃ for 2 hr. The absorbance (BMG LABTECH, Durham, NC, USA) was measured at 450 nm.

2.4. NO production assay

RAW 264.7 cells were plated in a 96-well plate (2×10^5 cells/well) and incubated for 24 hr. Cultured cells were treated with LPS (1 μg/mL) in the presence of different concentrations of sulforaphene (0~20 μM) and incubated for 24 hr. 100 μL of the culture media was mixed with an equal volume of Griess reagent (0.1% N-1-naphthylenediamine dihydrochloride, 2.5% phosphoric acid, and 1% sulfanilamide). The absorbance was measured at 540 nm. A sodium nitrite was used as a standard (31).

2.5. PGE_2 production assay

RAW 264.7 cells were cultured in a 6-well plate at a density of 1×10^6 cells/well for 24 hr. Different concentration of sulforaphene (0~10 μM) was added to the medium and maintained for 1 hr. And then it was stimulated by LPS (1 μg/mL) for 24 hr. The supernatant was collected and stored at -70℃. The level of PGE_2 in the culture media was quantitated by enzyme immuno assay kit (GE healthcare, Buckinghamshire, UK).

2.6. Pro-inflammatory cytokines production assay

RAW 264.7 cells were cultured in a 6-well plate at a density of 1×10^6 cells/well for 24 hr. Different concentration of sulforaphene (0~10 μM) was added to the medium and maintained for 1 hr. And then it was stimulated by

LPS (1 μg/mL) for 24 hr. The supernatant was collected and stored at -70℃. The levels of TNF-α, IL-1β, and IL-6 in the culture media were quantitated by ELISA assay kit (ELISA, R&D System, Minneapolis, MN, USA).

2.7. Immunoblot analysis

RAW 264.7 cells (1 × 10^6 cells/well) were incubated with different concentrations of sulforaphene (0～10 μM) for 1 hr and stimulated with LPS (1 μg/mL) for 20 min (NFκ-B), 10～50 min (Iκb-α), or 24 hr (iNOS and COX-2), respectively. The cells were washed twice with PBS and lysed with 1X RIPA lysis buffer containing 1 mM PMSF, Na_3VO_4, and NaF, and 1 μg/mL aprotinin, leupeptin, and pepstatin on ice for 1 hr, and then they were centrifuged at 12,000 rpm for 20 min at 4℃. Cytoplasmic and nuclear proteins were extracted using NE-PERTM nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL, USA). The protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of the protein (30～40 μg) were separated by 8, 10, or 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly vinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA, USA). The membrane was blocked with 5% skim milk powder in Tris buffered saline with 0.1% Tween 20 (TBS-T) at room temperature for 1 hr. After overnight incubation at 4℃ with the specific primary antibody, it was washed three times with TBS-T and incubated for 2 hr with a peroxidase-conjugated secondary antibody at room temperature. The immunoreactive bands were determined using enhanced chemiluminescence (ECL) and quantitated by TotalLab Quant v13 program (TotalLab Ltd., Newcastle upon Tyne, UK).

2.8. Total cellular RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) assay

RAW 264.7 cells were cultured in a 6-well plate for 24 hr and pretreated with different concentrations of sulforaphene (0~10 μM) for 1 hr and then stimulated with LPS (1 μg/mL) for 6 hr. Total cellular RNA was isolated with a TRI reagent (Molecular Research Center, cincinnati, OH, USA). One microgram of total RNA was reverse-transcribed using the ImProm-IITM Reverse Transcription System (Promega Corp., Madison, WI, USA) with an oligo dT-15 primer, as recommended by the supplier. The PCR assay was then carried out aliquots of the cDNA preparation in order to detect mRNA expression for iNOS, COX-2, TNF-α, IL-1β, IL-6 and β-actin. The reactions were conducted in a 25 μL volume containing 1 μL of Taq DNA polymerase, 0.2 mM dNTP, 10 reaction buffers, and 100 pmol of 5′ and 3′ primers. The cycle number was optimized to ensure product accumulation within the extension range. β-Actin was used as an internal control for normalizing the RNA content of each sample. After amplification, portions of the PCR reactions were electrophoresed on a 2% agarose gel and visualized under UV irradiation after ethidium bromide staining. Relative band density was determined by densitometry using image acquisition and analysis software (LabWorks, UVP, Cambridge, UK). Twenty to 25 cycles were used for iNOS, COX-2, TNF-α, IL-1β, IL-6 and β-actin.

2.9. Transient transfection and luciferase assay

RAW 264.7 cells were co-transfected with 1 mg of the NF-κB-promoted luciferase reporter gene plasmid pGL4.32[*luc2P*/NF-κB-RE/Hygro] (Promega, Madison, WI, USA) and 0.4 mg of the *Renilla* luciferase reporter plasmid pGL4.74[*hRluc*/TK] (Promega), which used as the internal standard, using

FuGENE 6 transfection reagent (Roche, Indianapolis, IN, USA). After 24 hr, the cells were treated with LPS (500 ng/mL) and incubated for 24 hr in the absence or presence of a sulforaphene. Luciferase activity in the cell lysate was determined using the Dual-Luciferase Reporter Assay Kit (Promega). The luciferase activity was normalized to the transfection efficiency, as monitored by the *Renilla* luciferase reporter plasmid expression vector. The level of induced luciferase activity was determined as a ratio to the luciferase activity of non-stimulated cells.

2.10. Statistical analysis

The data were expressed as means±SD with three replications. The statistical significance was tested using a multiple range test (Duncan's test, SPSS Inc., Chicago, IL, USA). $P \leq 0.05$ was considered to indicate statistical significance.

3. RESULTS AND DISCUSSION

3.1. Cytotoxicity of sulforaphene

The cytotoxicity of sulforaphene in RAW 264.7 cells was determined at different concentrations of 0~20 μM (Fig. 2). Sulforaphene had no effects on the viability of RAW 264.7 cells at less than 10 μM. However at the concentration of 20 μM, the cytotoxicity was 70%. Therefore the sulforaphene concentration of 0~10 μM was selected for further experiments.

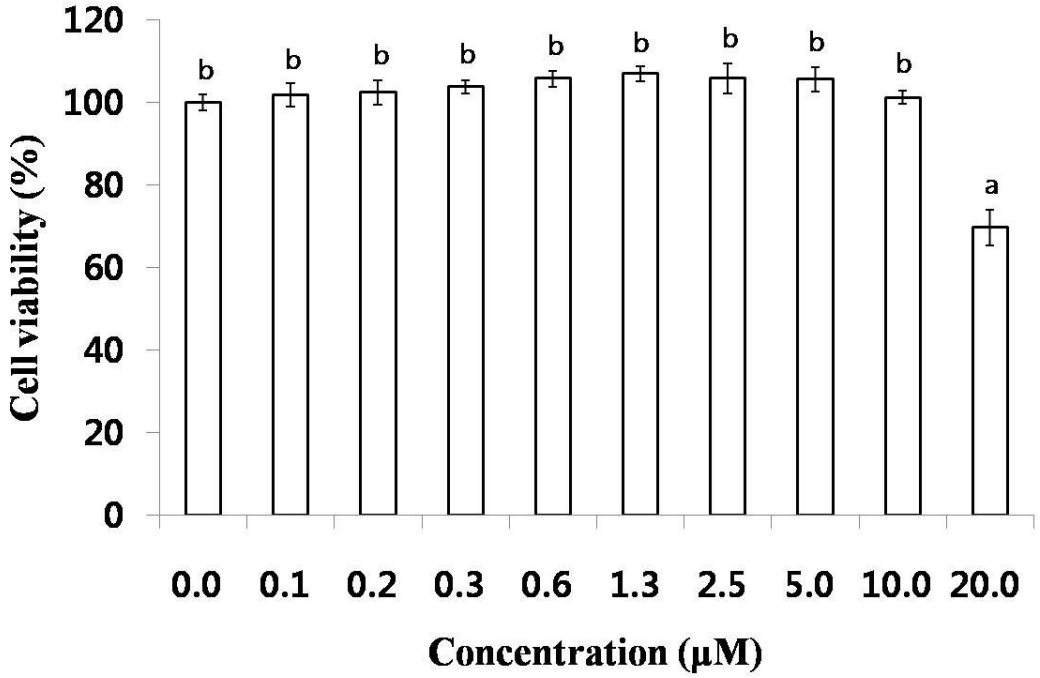


Fig. 2. The viability of LPS-stimulated RAW 264.7 cells with different concentration of sufloraphene. The values with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

3.2. Inhibitory effect of sulforaphene on LPS-stimulated NO production

The inhibitory effect of sulforaphene on NO production as a result product of inflammatory reaction was shown in Fig. 3. NO was produced by 20 μM in LPS-treated RAW 264.7 cells. Sulforaphene reduced NO levels in a dose-dependent manner in LPS-stimulated RAW 264.7 cells. The inhibition activity of sulforaphene against NO production was high as 91% compared to curcumin (80%) and pyrrolidine dithiocarbamate (PDTC) (12%) as the positive controls at the concentration of 10 μM. The 50% inhibition concentration (IC_{50}) of sulforaphene against NO production was low as 0.6 μM compared to sulforaphane (0.7 μM) which was well known as an active compound (32). Sulforaphene is different from sulforaphane in terms of the chemical structure where sulforaphene has a double bond in the alkyl chain.

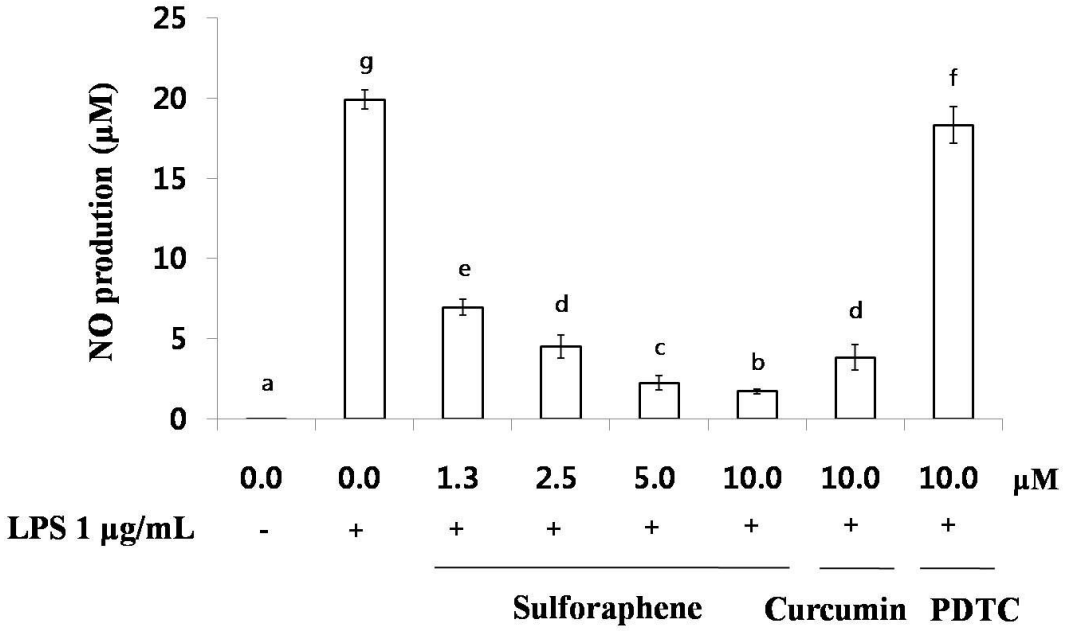


Fig. 3. Inhibitory effects of sulforaphene on NO production in LPS-stimulated RAW 264.7 cells. Curcumin and pyrrolidine dithiocarbamate (PDTC) were used as positive controls. The values with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

3.3. Inhibitory effect of sulforaphene on LPS-stimulated PGE_2 production

In inflammation processes, PGE_2 is involved in the classic signs of redness, heat, swelling, and pain (33). To examine whether sulforaphene could suppress PGE_2 production in LPS-stimulated macrophage, we measured PGE_2 levels in the cultured media added with different concentrations of sulforaphene (0~10 μM) in RAW 264.7 cells stimulated by LPS (1 μg/mL) (Fig. 4). Sulforaphene did not inhibit PGE_2 production, whereas curcumin (19%) and PDTC (6%) inhibited PGE_2 production induced by LPS. Meanwhile, sulforaphane from broccoli showed inhibitory activity against PGE_2 production with IC_{50} value of 1.4 μM (32).

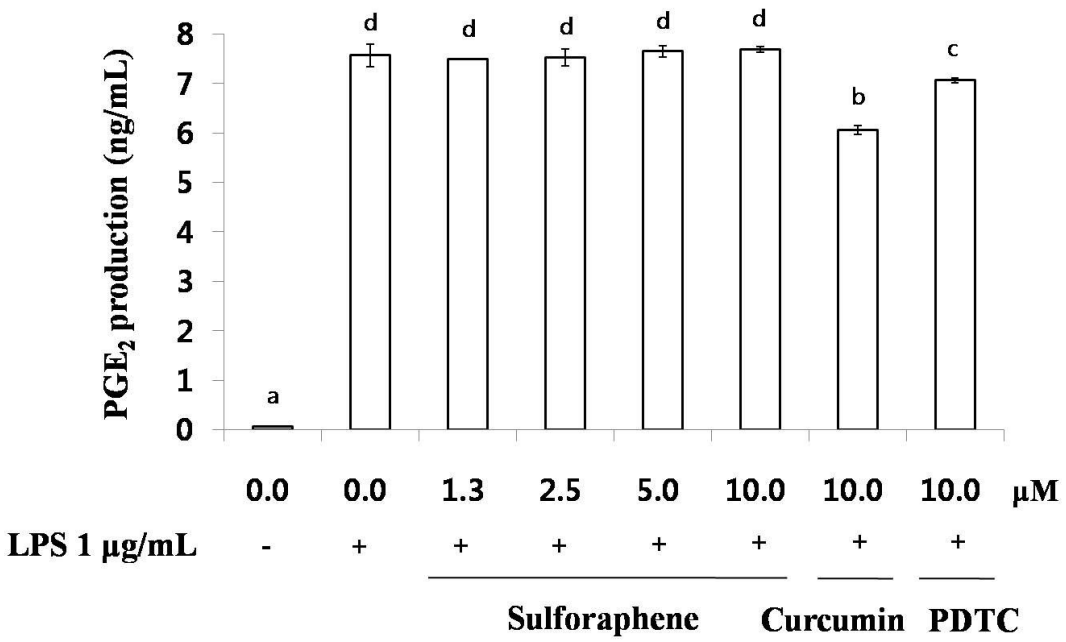
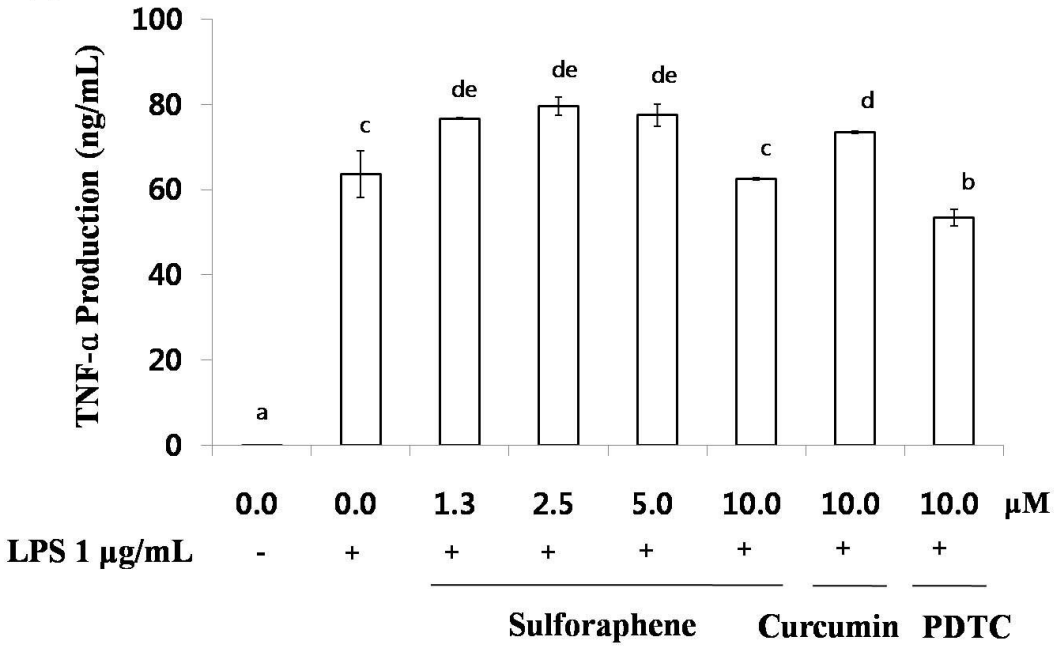


Fig. 4. Inhibitory effects of sulforaphene on PGE_2 production in LPS-stimulated RAW 264.7 cells. Curcumin and pyrrolidine dithiocarbamate (PDTC) were used as positive controls. The values with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

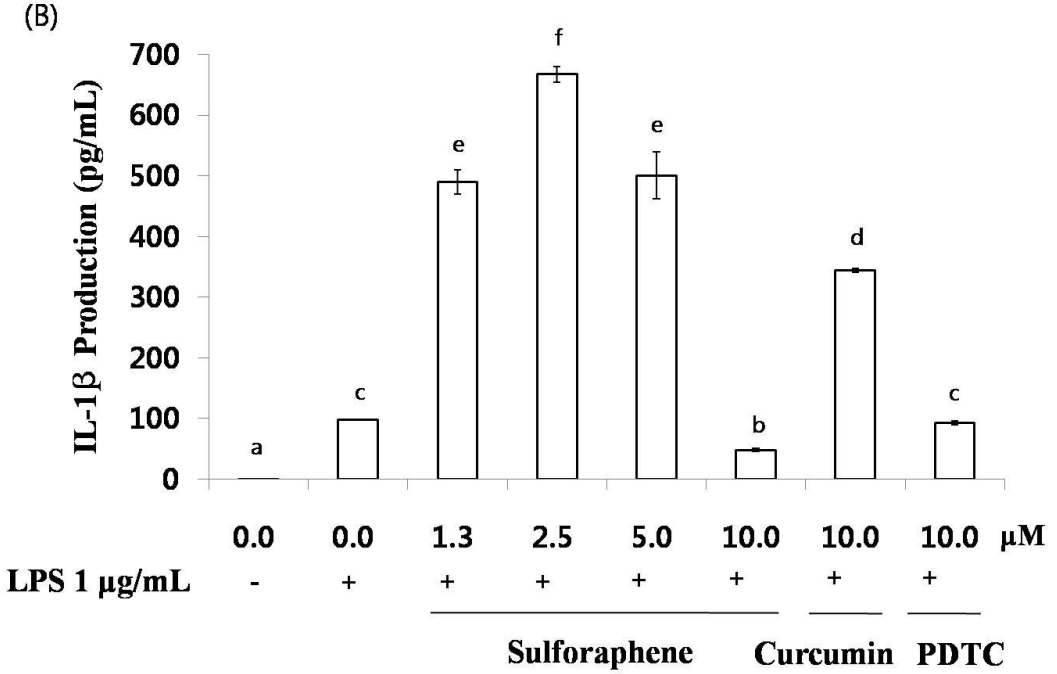
3.4. Inhibitory effect of sulforaphene on LPS-stimulated pro-inflammatory cytokines production (TNF-α, IL-1β, and IL-6)

LPS is endotoxin, activates the macrophage, and induces the secretion of pro-inflammatory cytokines such as TNF-a, IL-1β, and IL-6. TNF-α is released by stimulated cells and is an important mediator of shocklike syndrome, and causes fever and hypotension. TNF-α induces the cells to secrete IL-1β and IL-6, and IL-6 induces production of acute-phase proteins (34). Inhibitory effect of sulforaphene on pro-inflammatory cytokines production such as TNF-α, IL-1β, and IL-6 in macrophage supernatant was determined (Fig. 5). Sulforaphene did not inhibit TNF-α production (Fig. 5(A)) in this study. Meanwhile, sulforaphane from broccoli unlike sulforaphene from radish inhibited TNF-α production with IC_{50} values of 7.8 μM (32). Sulforaphene promoted the production of IL-1β at less than 5 μM, but slightly decreased it at 10 μM (Fig. 5(B)). In case of IL-6, LPS treatment increased the secretion of IL-6 by approximately 30 ng/mL (Fig. 5(C)). However, sulforaphene down-regulated the secretion of IL-6 in a concentration-dependent manner. In particular, IL-6 production in the culture supernatant treated with 10 μM of sulforaphene was completely inhibited as the same as the LPS-untreated group.

(A)



(B)



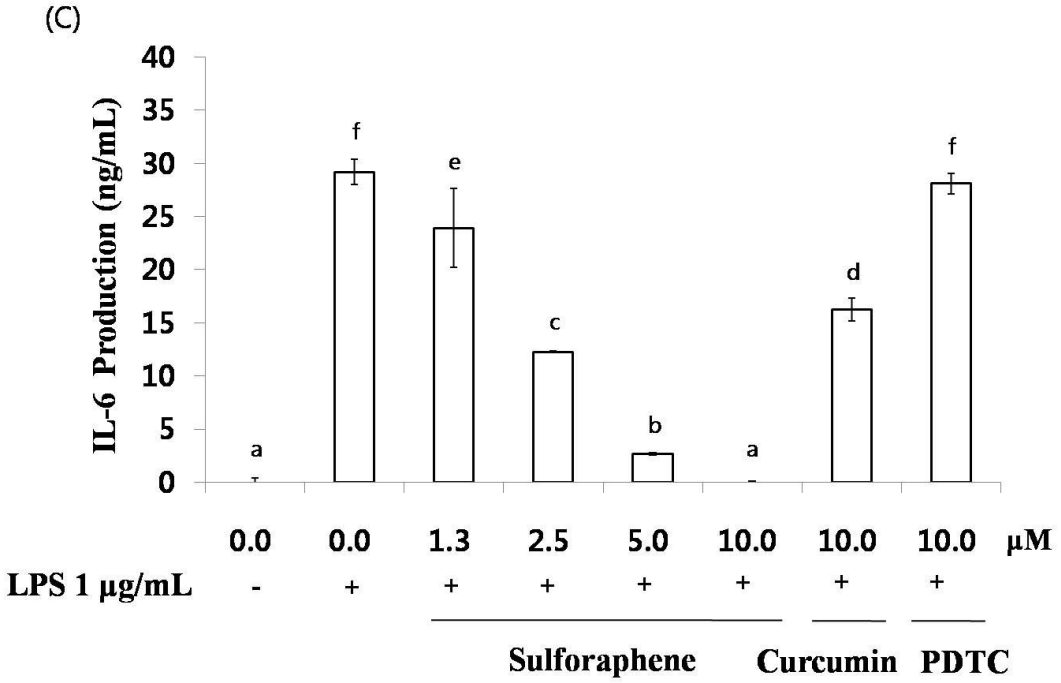


Fig. 5. Inhibitory effects of sulforaphene on TNF-α (A), IL-1β (B), and IL-6 (C) production in LPS-stimulated RAW 264.7 cells. Curcumin and pyrrolidine dithiocarbamate (PDTC) were used as positive controls. The values with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

3.5. Inhibitory effect of sulforaphene on LPS-stimulated iNOS and COX-2 protein expressions

In this study, we found that sulforaphene significantly inhibited LPS-induced NO production, but didn't inhibit PGE_2 production. NO and PGE_2 are produced by iNOS and COX-2 enzymes, respectively. Thus expressions of iNOS and COX-2 proteins were measured by western blotting whether the inhibitory effect of sulforaphene on NO and PGE_2 production is caused by inhibition of iNOS and COX-2 enzymatic activities (Fig. 6).

LPS treatment significantly induced iNOS protein expression compared to the LPS-untreated, whereas sulforaphene effectively inhibited the expression of iNOS protein in a concentration-dependent manner. 1.25 μM of sulforaphene lowered iNOS protein by 88%, and 5-10 μM of sulforaphene completely suppressed induction of iNOS protein. Curcumin also completely inhibited iNOS expression, and PDTC inhibited more than 52% at 10 μM of concentration. It was reported that 1.5 μM of sulforaphane from broccoli inhibited 61% of iNOS protein (32).

Meanwhile, sulforaphene did not inhibit COX-2 enzyme expression induced by LPS treatment, whereas COX-2 enzyme was slightly inhibited by curcumin.

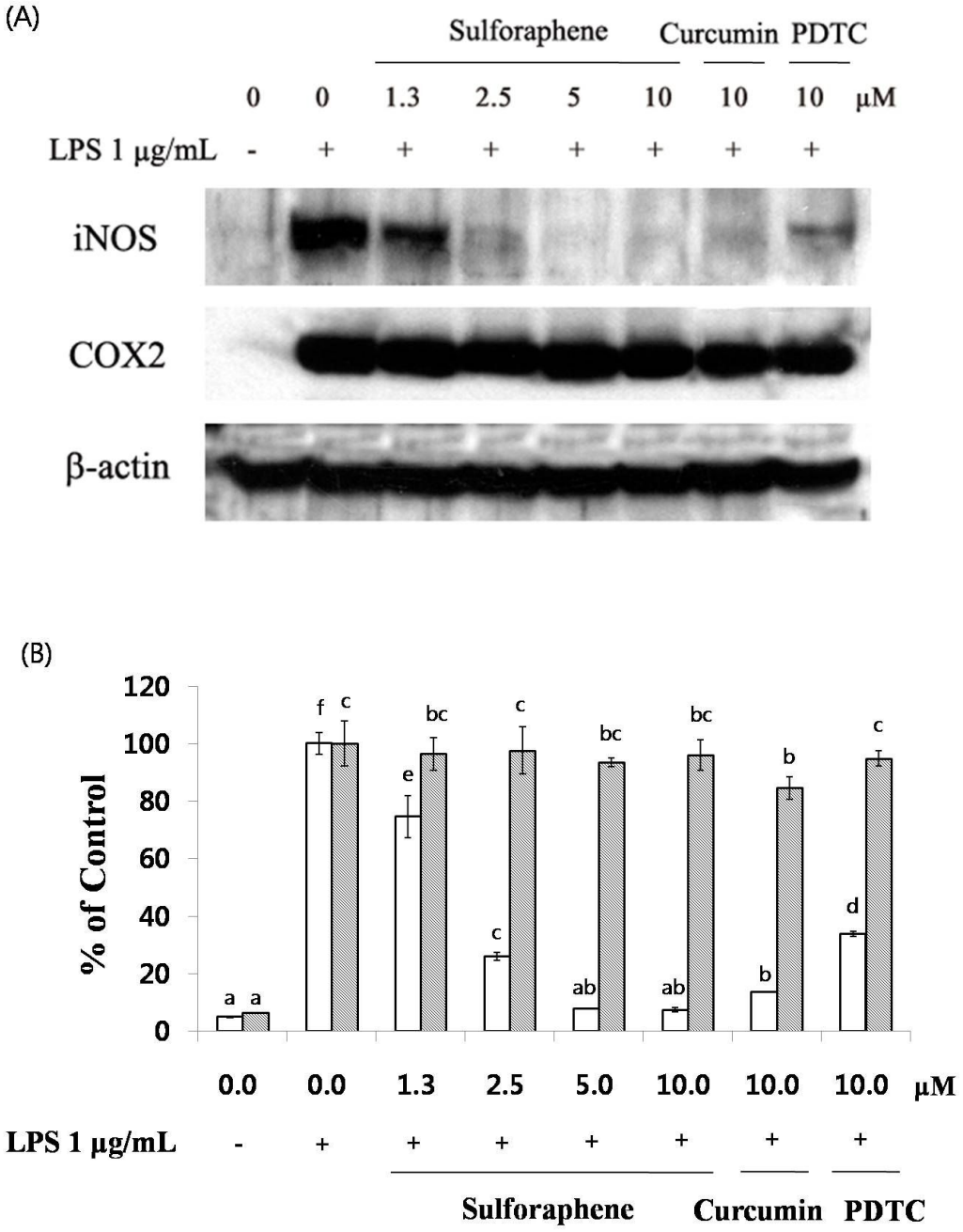


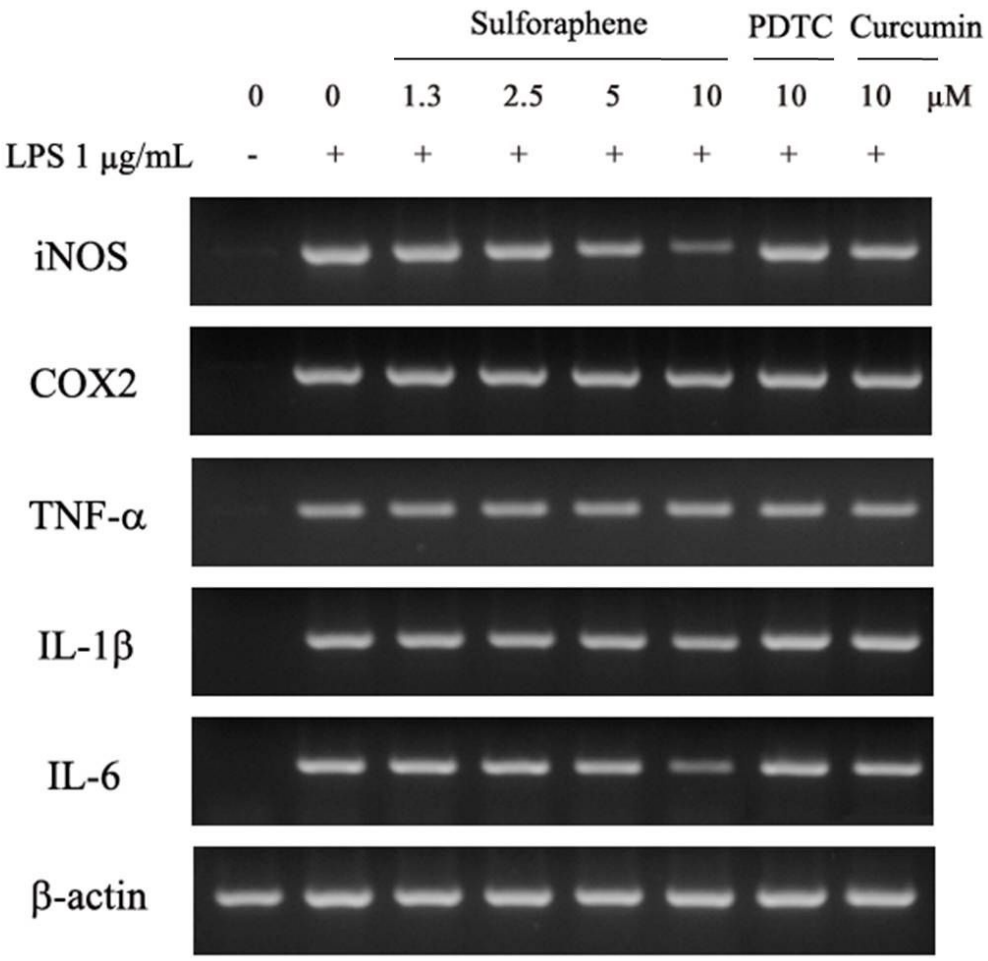
Fig. 6. Inhibitory effects of sufloraphene on iNOS (□) and COX-2 (▨) protein expressions in LPS-stimulated RAW 264.7 cells. Curcumin and pyrrolidine dithiocarbamate (PDTC) were used as positive controls. The values with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

3.6. Inhibitory effect of sulforaphene on LPS-stimulated mRNA expression

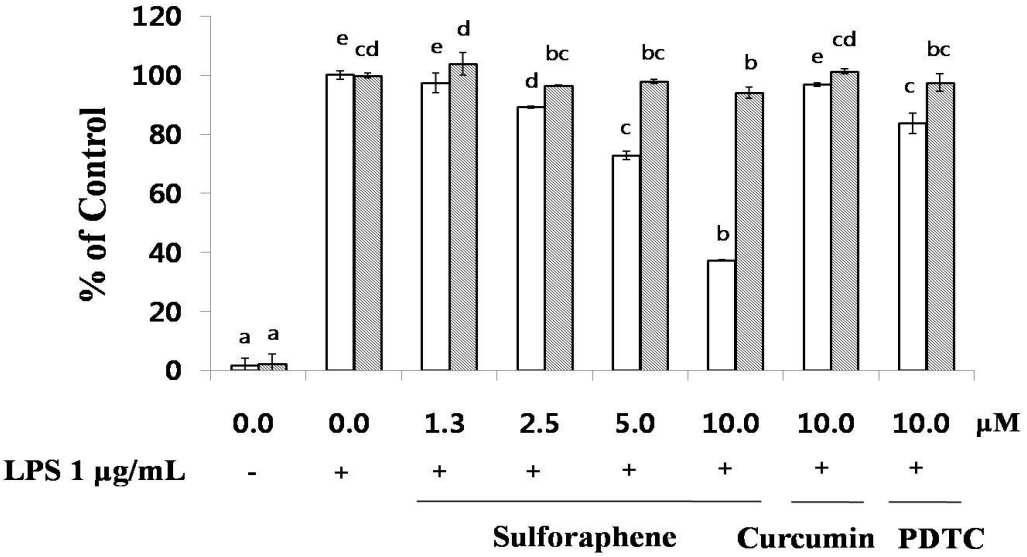
Pro-inflammatory mediators such as iNOS, COX-2, TNF-α, IL-1β, and IL-6 mRNA is up-regulated by LPS treatment in RAW 264.7 cells. We investigated the effect of sulforaphene on mRNA transcription (Fig. 7). Sulforaphene dramatically reduced iNOS mRNA in a concentration-dependent manner. In particular, sulforaphene inhibited iNOS mRNA by 65%, and curcumin inhibited by 16% at 10 μM, but PDTC did not inhibit (Fig. 7(B)). Meanwhile, COX-2 (Fig. 6(B)) and cytokines including TNF-α and IL-1β mRNA (Fig. 6(C)) was not inhibited by sulforaphene. Only IL-6 mRNA was inhibited by sulforaphene (Fig. 6(C)). 10 μM of sulforaphene blocked roughly 50% of IL-6 mRNA generated by LPS treatment.

Therefore, the inhibition of sulforaphene on NO and IL-6 production resulted from the inhibition of iNOS and IL-6 mRNA transcription, respectively. In addition, no inhibitory effects of the production of COX-2 and cytokines including TNF-α and IL-1β in Fig. 4 and 5 coincided with the results of mRNA expressions in Fig. 7. It was concluded that sulforaphene could regulate inflammatory responses through the inhibition of iNOS and IL-6 pathway.

(A)



(B)



(C)

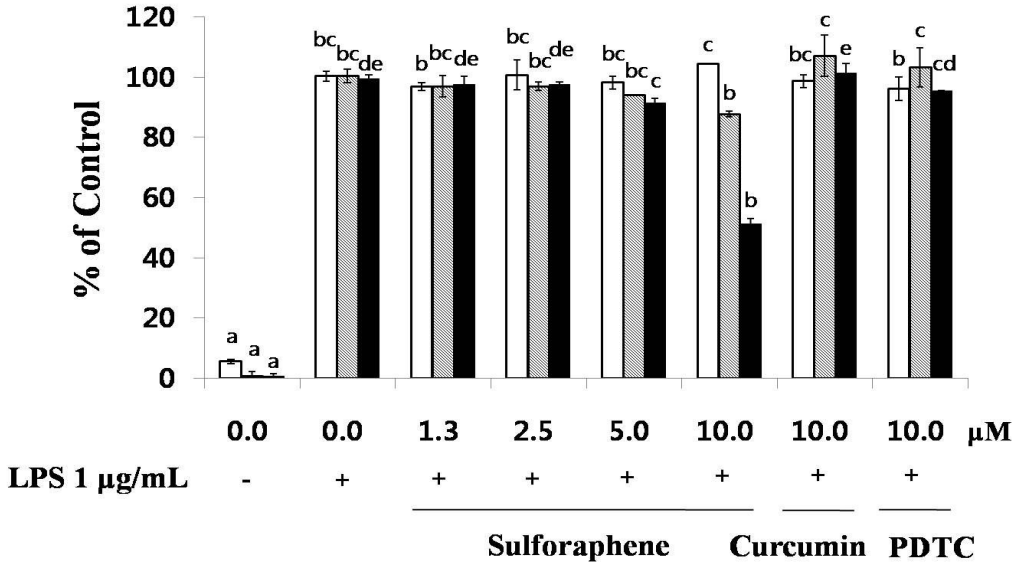


Fig. 7. Inhibitory effects of sulforaphene on mRNA expression in LPS-stimulated RAW 264.7 cells. Curcumin and pyrrolidine dithiocarbamate (PDTC) were used as positive controls. The values with different letters are significantly different at $p<0.05$ by Duncan's multiple range test. ((B) □: iNOS, ▨: COX-2, (C) □: TNF-α, ▨: IL-1β, ■: IL-6).

3.7. Inhibitory effect of sulforaphene on NF-κB transcriptional activity

When NF-κB moved into nucleus, NF-κB binds to target DNA binding site and causes transcription of genes encoding proteins associated with inflammation. The NF-κB transcriptional activity was measured by luciferase assay (Fig. 8). Treatment of LPS increased NF-κB activity by 20-fold. Sulforaphene caused a dose-dependent inhibition of NF-κB transcriptional activity. At the concentration of 10 μM, NF-κB activity was similar to LPS-untreated. Curcumin and PDTC lowered NF-κB transcriptional activity by 60 and 30%, respectively.

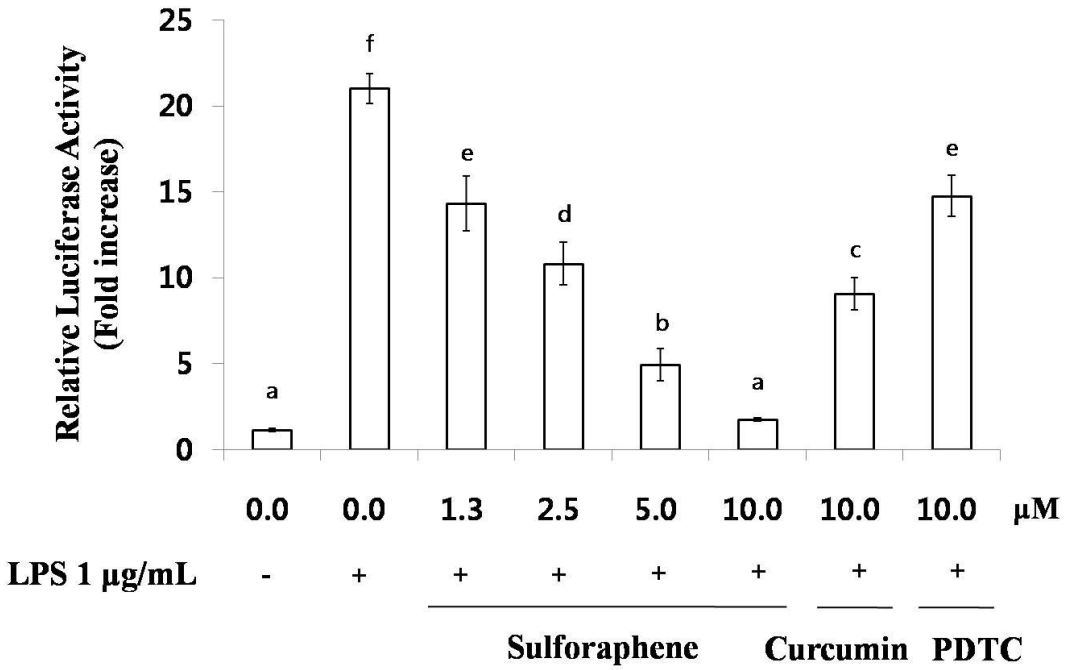


Fig. 8. Inhibitory effects of sulforaphene on luciferase activity in LPS-stimulated RAW 264.7 cells with sulforaphene. Curcumin and pyrrolidine dithiocarbamate (PDTC) were used as positive controls. The values with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

3.8. Inhibitory effect of sulforaphene on degradation of Iκb-α and activation of NF-κB

Producrion of NO and PGE_2 by iNOS and COX-2, and secretion of pro-inflammatory cytokines are regulated by NF-κB pathway (25, 26). NF-κB plays a crucial role in regulation of inflammation in LPS-stimulated macrophage. NF-κB is activated and translocated to the nucleus when phosphorylation and degradation of Iκb-α occur by LPS. Then NF-κB induces gene related pro-inflammatory mediators (27). Thus suppression effects of sulforaphene on degradation of Iκb-α and activation of NF-κB were evaluated in LPS-stimulated RAW 264.7 cells. First, to confirm the degradation time of Iκb-α by LPS, Iκb-α expression was measured at 0~50 min depending on exposure time of LPS in the presence or absence of sulforaphene (Fig. 9). Sulforaphene inhibited degradation of Iκb-α at 0~50 min treated in this study. However, the non-treated group with sulforaphene has initiated degradation of Iκb-α within 10 min and completely degraded after 20 min. Thus, Iκb-α and NF-κB proteins were measured in cytoplasm and nucleus, respectively after 20 min treatment of LPS (Fig. 10). When LPS was not treated, Iκb-α remained in cytoplasm. In the case of LPS treatment, on the other hand, Iκb-α was not detected at 0-5 μM but exists slightly at 10 μM of sulforaphene in cytoplasm. Meanwhile, NF-κB was translocated in nucleus in the presence of LPS. Sulforaphene inhibited the translocation of NF-κB into the nucleus in a concentration-dependent manner, and NF-κB did not exist at 10 μM of sulforaphene in nucleus. These results suggest that sulforaphene inhibits inflammatory processes via inhibition on degradation of I κb-α within 20 min.

It was concluded that inhibitory effect of sulforaphene on production of iNOS and IL-6 was regulated mainly by NF-κB in LPS-stimulated macrophages.

Excessive NO produced by iNOS is a vascular permeability factor and brings about mutagenesis, and tissue damage, tumor growth (35). IL-6 is also a pro-inflammatory cytokine mostly synthesized by macrophages and causes rheumatoid arthritis and atherosclerosis (36, 37). Thus, we suggest that sulforaphene may be used to treat inflammatory disorders, rheumatoid arthritis and atherosclerosis.

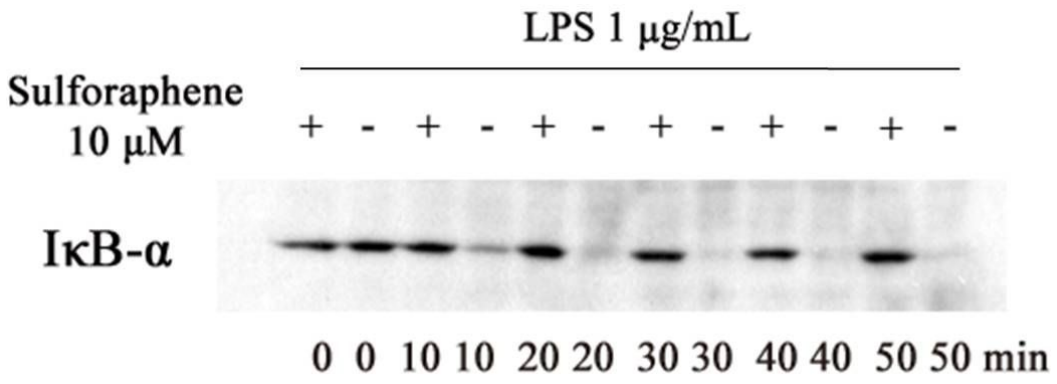


Fig. 9. Inhibitory effects of sulforaphene on degradation of Iκb-α depending on exposure time of LPS in RAW 264.7 cells. The values with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

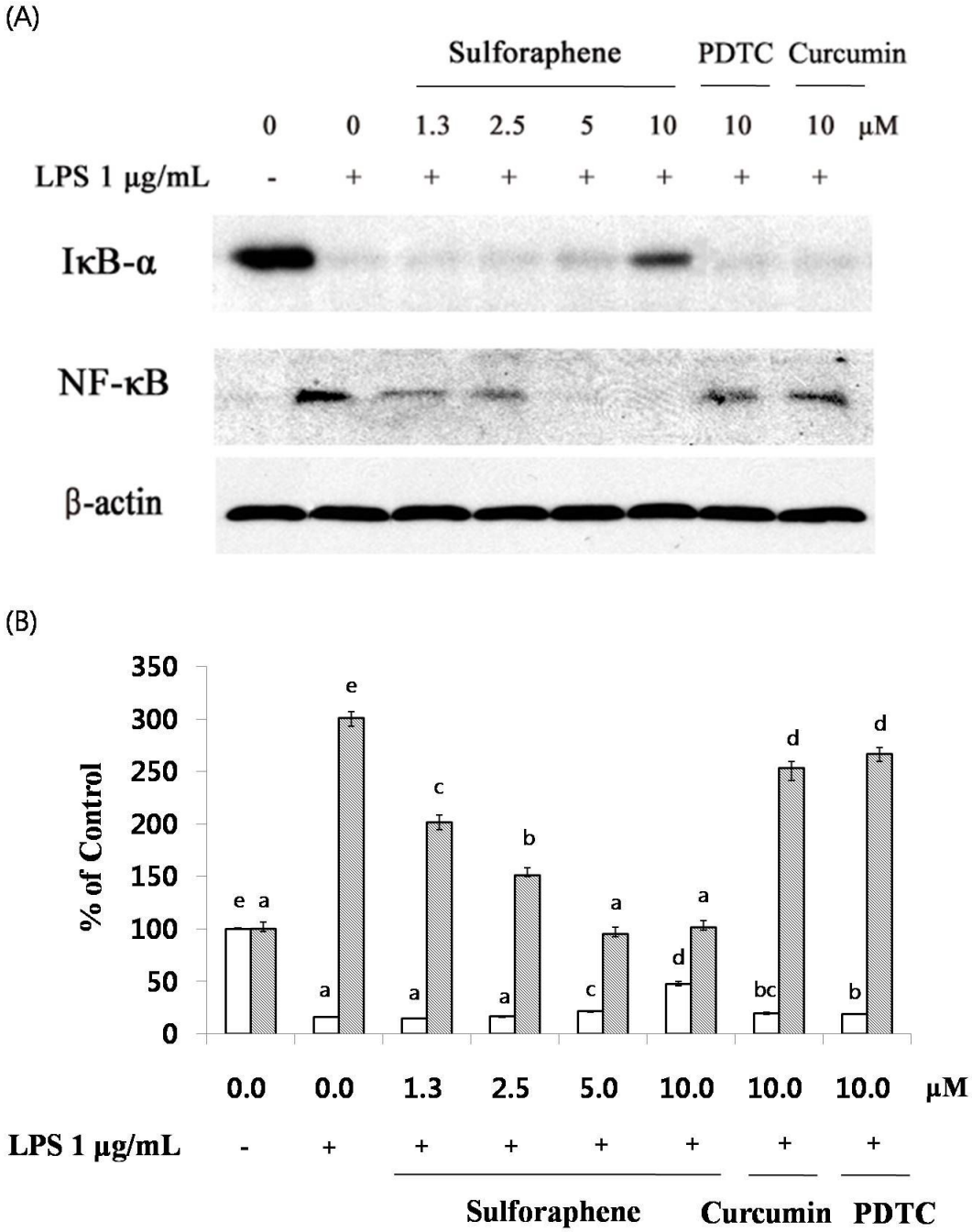


Fig. 10. Inhibitory effects of sulforaphene on degradation of Iκb-α (□) and activation of NF-κB (▨) in LPS-stimulated RAW 264.7 cells. Curcumin and pyrrolidine dithiocarbamate (PDTC) were used as positive controls. The values with different letters are significantly different at $p<0.05$ by Duncan's multiple range test.

REFERENCES

1. Gutierrez RMP and Perez RL. *Raphanus sativus* (Radish): Their chemistry and biology. The scientific world journal 4: 811-837 (2004)

2. Abdou IA, Abou-Zeid AA, El-Sherbeeny MR, Abou-El-Gheat ZH. Antimicrobial activities of *allium sativum*, *allium cepa*, *raphanus sativus*, *capsicum frutescens*, *eruca sativa*, *allium kurrat* on bacteria. Qual. Plant. Mater. Veg. 22: 29-35 (1972)

3. Lugasi A, Dworschak E, Blazovics and Kery A. Antioxidant and free radical scavenging properties of squeezed juice from black radish (*Raphanus sativus L.* var *niger*) root. *Phytother. Res.* 12: 502-506 (1998)

4. Nakamura Y, Iwahashi T, Tanaka A, Koutani J, Matsu T, Okamoto S, Sato K and Ohtsuki K. 4-(Methylthio)-3-butenyl isothiocyanate, a principal antimutagen in daikon (Raphanus sativus; Japanese white radish). J. Agric. Food Chem. 49: 5755-5760 (2001)

5. Hanlon PR, Robbins MG, Hammon LD and Barnes DM. Aqueous extract from the vegetative portion of Spanish black radish (*Raphanus sativus* L. var. *niger*) induces detoxification enzyme expression in HepG2 cells. J. Funct. Foods 1: 356-365 (2009)

6. Pocasap P, Weerapreeyakul N and Barusrux S. Cancer preventive effect of Thai rat-tailed radish (*Raphanus sativus* L. var. *caudatus* Alef). J. Func. Foods 5: 1372-1381 (2013)

7. Jakubikova J, Bao Y and Sedlak J. Isothiocyanates induce cell cycle arrest, apoptosis and mitochondrial potential depolarization in HL-60 and multidrug-resistant cell lines. Anticancer research 25: 3375-3386 (2005)

8. Papi A, Orlandi M, Bartolini G, Barillari J, Iori R, Paolini M, Ferrini F, Fumo MG, Pedulli GF and Valgimigli L. Cytotoxic and antioxidant activity of 4-methylthio-3-butenyl isothiocyanate from *Raphanus sativus* L. (Kaiware Daikon) sprouts. J. Agric. Food Chem. 56: 875-883 (2008)

9. Nastruzzi C, Cortesi R, Esposito E, Menegatti E, Leoni O, Iori R and Palmieri S. *In vitro* cytotoxic activity of some glucosinolate-derived products generated by myrosinase hydrolysis. J. Agric. Food Chem. 44: 1014-1021 (1996)

10. Nastruzzi C, Cortesi R, Esposito E, Menegatti E, Leoni O, Iori R and Palmieri S. In vitro antiproliferative activity of isothiocyanates and nitriles generated by myrosinase-mediated hydrolysis of glucosinolates from seeds of cruciferous vegetables. J. Agric. Food Chem. 48: 3572-3575 (2000)

11. Shishu and Kaur IP. Inhibition of cooked food-induced mutagenesis by dietary constituents: Comparison of two natural isothiocyanates. Food Chem. 112: 977-981 (2009)

12. Nathan C. Points of control in inflammation. Nature 420: 846-852 (2002)

13. Mantovanil A, Allavena P, Sica A and Balkwill F. Cancer-related inflammation. nature 454: 436-444 (2008)

14. Dandona P, Aljada A and Bandyopadhyay A. Inflammation: the link

between insulin resistance, obesity and diabetes. *Trends immunol.* 25: 4-7 (2004)

15. Tracey KJ. The inflammatory reflex. Nature 420: 853-859 (2002)

16. Raetz CRH and Whitfield C. Lipopolysaccharide endotoxins. Annu. Rev. Biochem. 71: 635-700 (2002)

17. Vane JR, Mitchell JA, Tomlinson A, Bishop-Bailey D, Croxtall J and Willoughby DA. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. Proc. Natl. Acad. Sci. USA 91: 2046-2050 (1994)

18. Willeaume V, Kruys V, Mijatovic T and Huez G. Tumor necrosis factor-alpha production induced by vuruses and by lipopolysaccharides in macrophages: similarities and differences. J. inflamm. 46: 1-12 (1995-1996)

19. Rogler G and Andus T. Cytokines in inflammatory bowel disease. World J. Surg. 22: 382-389 (1998)

20. Knowles RG and Moncada S. Nitric oxide synthases in mammals. Biochem. J. 298: 249-258 (1994)

21. Aktan Fugen. iNOS-mediated nitric oxide production and its regulation. Life Sciences 75: 639-653 (2004)

22. Bennett A, Tacca D, Stamford IF and Zebro T. Prostaglandins from tumours of human large bowel. Br. J. Cancer 35: 881-884 (1977)

23. Bennettt A, Carroll MA, Stamford IF, Whimster WF and Williams F.

Prostaglandins and human lung carcinomas. Br. J. Cancer 46: 888-893 (1982)

24. Bennett A, Stamford IF, Berstock DA, Dische F, Singh L and A'Hern RP. Breast cancer, prostaglandins and patient survival. Br. J. Cancer 59: 268-275 (1989)

25. Balckwell TS and Christman JW, The role of nuclear factor-κB in cytokine gene regulation. Am. J. Respir. Cell Mol. Biol. 17: 3-9 (1997)

26. Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK and Lee SS. Molecular mechanism underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activiation. Mutation Research 480-481: 243-268 (2001)

27. Simmonds RE and Foxwell. NF-κB and its relevance to arthritis and inflammation. Rheumatology 47: 584-590 (2008)

28. Ghazizadeh M. Essential role of IL-6 signaling pathway in keloid pathogenesis. J. Nippon Med. Sch. 74: 11-22 (2007)

29. Tak PP and Firestein GS. NF-κB: a key role in inflammatory diseases. J. Clin. Invest. 107: 7-11 (2001)

30. Li BL, Wang FQ, Gao MM, Pan M, Tan J and Shan YJ. The study on the effect of sulforaphene homologue on the inhibition of COX-2 expression. Information on Traditional Chinese Medicine (2011)

31. Snell JC, Colton CA, Chernyshev ON and Gilbert DL. Location-dependent artifact for NO measurement using multiwell plates. Free Radical Bio. Med. 20: 361-363 (1996)

32. Heiss E, Herhaus C, Klimo K, Bartsch H and Gerhauser C. Nuclear factor-κB is a molecular target for sulforaphnae-mediated anti-inflammatory mechanisms. J. Biol. Chem. 276: 32008-32015

33. Ricciotti E and FitxGerald GA. Prostaglandins and inflammation. Arterioscler. Thromb. Vasc. Biol. 31: 986-1000 (2011)

34. Jean-Baptiste E. Cellular mechanisms in sepsis. J. Intensive Care Med. 22: 63-72 (2007)

35. Maeda H and Akaike T. REVIEW: Nitric oxide and oxygen radicals in infection, inflammation, and cancer. Biochem. 63: 854-865 (1998)

36. Srirangan S and Choy EH. The role of interleukin in the pathophysiology of rheumatoid arthritis. Ther. Adv. Musculoskel. Dis. 2: 247-256 (2010)

37. Amar J, Fauvel J, Drouet L, Ruidavets JB, Perret B, Chamontin B, Boccalon H and Ferrieres J. Interleukin 6 is assoiated with subclinical atherosclerosis: alink with soluble intercellular adhesion molecule 1. J. Hypertens. 24: 1083-8 (2006)

ACKNOWLEDGMENT

제게 있어 학위과정의 끝은 학문의 길 보다는 어쩌면 인격수양의 과정에 더 가깝지 않았나 싶습니다. 공부를 하며 많은 고민들로 한계를 느끼기도 했었지만, 이제 비로소 모든 과정을 마치고 논문의 마지막 마무리를 글로 남기려 하니 옛일이 스쳐 지나가면서 베풀지 못하고 받기만 한 삶을 반성하게 됩니다.

대학원 과정 가운데 배우며 감사한 분들이 많습니다.

학교에 입학했을 때의 제 모습과 현재의 제 모습을 비교한다면 많은 것이 변화되고 성장했음을 알 수 있습니다. 이와 같은 변화가 이뤄지게 된 바탕에는 지도교수님이신 임상빈 교수님의 가르침이 있었습니다. 많은 것들을 경험하고 배울 수 있도록 배려 해주셨고, 연구자로서의 태도와 학습방법을 터득할 수 있게 하셨습니다. 부족하고 실수투성이인 저를 매번 이끌어주셨는데 그에 끝까지 따라가지 못한 것 같아 죄송스런 마음뿐입니다. 진심으로 감사한 마음을 갖고 살아가겠습니다.

학부 때부터 석사 과정 마치기까지 많은 가르침을 주시고 조언을 해주신 하진환 교수님, 고영환 교수님, 박은진 교수님과 대학원 생활 동안 열정적으로 가르쳐주신 김현정 교수님께 진심으로 감사드립니다.

저와 대학원 생활을 같이하면서 제 실험 핑계로 많은 도움 못 드렸지만, 오히려 저를 볼 때 마다 응원과 격려해주신 최영진 교수님과 강윤구 선생님 정말 감사합니다. 또한 실험실에서 제가 하지 못했던 일들을 도맡아 우리 실험실을 이끌어주고, 저의 고민을 함께 해준 이충우 연구원님, 재원이, 수미한테도 고마움을 전합니다.

제주대학교 생명과학기술혁신센터에서 대부분의 대학원 생활을 보냈는데 많은

도움을 주신 직원들께 감사드립니다. 특히 연구개발부에 부희정 팀장님과 황준호 과장님, 제 친구 이미란 연구원 덕분에 실험에서부터 논문을 완성하는데 많은 도움을 받았고, 옆에서 끊임없이 응원 반, 잔소리 반 해준 후배 정수열 연구원, 마주칠 때 마다 응원해주셨던 제주양채류RIS사업단에 김민정 선생님, 조윤숙 선생님 너무 감사했습니다. 그리고 제 대학원 생활동안 저 때문에 저보다 더 스트레스 받았던 수환이한테도 고마움을 전합니다.

마지막으로 2년 동안 공부한다는 핑계로 매일 늦게 들어가고, 집안일 돕지도 못한 저를 묵묵히 지켜보며 응원해준 저의 가족 아빠, 엄마, 제 동생 봉영이, 진영이 너무 감사하고 사랑합니다.

앞으로 저를 응원해준 모두에게 보탬이 되고 즐거움을 줄 수 있는 사람이 되도록 끊임없이 노력하고 정진하겠습니다.