Master's Thesis of Science in Biomodulation

The preventive effects of sulforaphene on the early stage of atherosclerosis and its stability in aqueous solution

설포라핀의 초기 동맥경화 예방 효과 및 수용액에서의 안정성 연구

August 2019

The Graduate School

Seoul National University

Biomodulation Major

Jiwoo Yu

ABSTRACT

Radish (*Raphanus sativus*) seed and sulforaphene have been used in herbal medicine for hypertension and inflammatory diseases. They are also known to have anti-cancer and anti-inflammatory effects. However, they have not studied that it has the preventive effects on cardiovascular disease.

In this study, we investigated the preventive effects of radish seed extract and sulforaphene, a major component of radish seed extract, against the early stage of atherosclerosis. Radish seed extract and sulforaphene suppressed the adhesion of THP-1 monocytes to endothelial cell. In addition, we demonstrated that sulforaphene significantly attenuated the expression of TNF-α-induced vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) involved in monocyte adhesion and migration. Sulforaphene also regulated the phosphorylation of IκB kinase (IKK) and NF-κB inhibitor (IκB) α and degradation of IκBα related to Nuclear Factor-

Kappa B (NF-κB) pathway in a concentration-dependent manner. Furthermore, we found that sulforaphene inhibited oxidized low-density lipoprotein (ox-LDL)-induced lipid uptake by macrophages and foam cell formation.

However, sulforaphene was unstable in aqueous solution. In this study, we confirmed the stability of sulforaphene in aqueous solution for 7, 15 and 30 days at -20°C, 4°C and 25°C. We found that sulforaphene was degraded in aqueous solution at 25°C and the inhibitory effects of sulforaphene on early atherosclerosis was decreased in the presence of water at 25°C in a time-dependent manner.

In conclusion, radish seed and sulforaphene are potential sources that can help prevent the early stage of atherosclerosis and the optimal storage temperatures are -20°C and 4°C. This study could provide a good guideline to the industry for sulforaphene storage and application.

Keywords: Sulforaphene, monocyte-endothelial cell adhesion, plaque, atherosclerosis prevention, stability;

Student ID: 2017-25119

CONTENTS

Ⅰ. INTRODUCTION

Atherosclerosis is related to the development of cardiovascular disease which is the leading cause of death worldwide[1]. Many studies have been suggested that inflammation plays an important role in the initiation of atherosclerosis, so it is important to control the inflammatory response for its prevention[2]. Increased expression of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) is involved in the expression of VCAM-1 which is the cell adhesion molecule[3, 4]. When monocytes are adhered to vascular endothelial cells, they enter the intima and differentiate into macrophages. The macrophages then take up the oxidized LDL and promote foam cell formation[5]. Ox-LDL results in plaque formation and abnormal growth of vascular smooth muscle cells which plays a crucial role in the process of atherosclerosis[6-8]. In order to prevent the early stage of atherosclerosis, it is important to inhibit monocyte adhesion to vascular endothelial cells.

Radish (*Raphanus sativus*) seed is a root vegetable of *Brassicaceae* and cultivated throughout the world. Radish has been traditionally used for the treatment of various diseases such as gastrointestinal and respiratory systems[9]. Especially, Radish (*Raphanus sativus*) seed has been used for anti-cancer and anti-inflammatory agents in traditional Korean medicine[10]. 4-methylthio-butanyl derivatives are one of the main compositions of radish seed[11]. Glucoraphenin, 4-methylsulfinyl-3-butenyl glucosinolate, is hydrolyzed by myrosinase to sulforaphene[12]. Many studies have investigated the antioxidant and anti-carcinogenic effects of radish seed and sulforaphene, but its preventive effects on monocyte-endothelial cell adhesion have not been studied. In this study, we investigated the effects of radish seed and sulforaphene on preventing the adhesion of THP-1 monocytes to endothelial cells in HUVECs and its molecular mechanism.

However, there is an issue that isothiocyanates are not stable during storage. Few studies have found the stability of sulforaphene in various solvent conditions and its degradation products[13]. Also, the stability in various pH conditions was reported and it was degraded at alkaline environment[14, 15]. In this study, we confirmed the stability of sulforaphene in aqueous solution.

II. MATERIALS AND METHODS

1. Chemicals and reagents

Radish seed extract was derived from Laboratory of Food-medicine genomics and sulforaphene was purchased from LKT labs (Saint Paul, MN, USA, ≥98%, HPLC). Ginkgo leaf extract as a control was derived from Korea plant extract bank (Cheongju, Republic of Korea). Medium199 (M199) and penicillin (10,000 units/ml)-streptomycin (10,000 μg/ml) (P/S) was purchased from Corning (Corning, NY, USA). Hydrocortisone, 2-mercaptoethanol, puromycin, fetal bovine serum (FBS) and calcein-AM dye were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium was purchased from Welgene (Daegu, Republic of Korea). Fetal bovine serum (FBS), basic fibroblast growth factor (bFGF), and recombinant human epidermal growth factor (hEGF) were purchased from Gibco (Grand Island, NY, USA). Recombinant human tumor necrosis factor-alpha (TNF-α) was purchased from PeproTech Korea (Seoul, Republic of Korea). Oxidized-low density lipoprotein (ox-LDL) from human plasma was purchased from Alfa aesar (Haverhill, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium salt (MTT) solution was purchased from USB Corporation (Cleveland, OH, USA). Dimethylsulfoxide (DMSO) was purchased from Duksan Pure Chemicals (Ansan, Republic of Korea). Antibodies against vascular cell adhesion molecule-1 (VCAM-1), β-actin and basal IκB kinase (IKK) α/β were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Phosphorylated IKKα/β and IκBα, basal IκBα, phosphorylated ERM and basal ERM were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). Moreover, the analytical HPLC equipment was Ultimate3000 HPLC (Thermo Dionex, USA) with an analytical reversed phase Inno C-18 column (250 mm x 4.6 mm x 5 μm, Youngjin biochrom, Korea).

2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Walkersville, MD, USA) and cultured in Medium 199 media with HEPES containing 10% (v/v) FBS (Gibco), 2 mM Lglutamine, 1 ng/ml EGF, 2 ng/ml bFGF, 1 ng/ml hydrocortisone and 1% (v/v) streptomycin/penicillin. HUVECs were grown in a 5% CO_2 incubator at 37°C and used between passages 6 and 14. Human monocytic leukemia cell line THP-1 was obtained from the Korean Cell Line Bank (KCLB) and grown in RPMI 1640 media supplemented with 10% (v/v) FBS, 0.05 mM 2-mercaptoethanol, 1% (v/v) streptomycin/penicillin. Density of THP-1 was maintained between 2 x 10^5 and 1 x 10^6 cells/ml.

3. Cell viability assay

HUVECs and RAW264.7 were seeded in 96-well plates and incubated with different concentrations of radish seed extracts and sulforaphene for 22 hours. After incubation, MTT solution was added to each well of 96-well plates to 0.5 mg/ml of medium. After 2 hours, the medium was removed and 200 μl of dimethyl sulfoxide (DMSO) was added. After 15 minutes of shaking, the absorbance was measured at wavelength of 570 nm by using 96-well microplate reader.

4. THP-1 monocyte adhesion assay

HUVECs were seeded in 96-well plates and pretreated with each concentrations of radish seed extracts and sulforaphene for 1 hour before stimulation with 10 ng/ml TNF-α. After 5 hours of incubation, calcein-AM-labeled THP-1 cells were added to HUVECs at density of 5 x 10^5 cells/well in M199. After 1 hour of incubation, non-adhered THP-1 cells were removed with phosphate-buffered saline (PBS) washing. Fluorescence of adhered THP-1 cells was determined by using an Infinite 200 PRO system (Tecan group Ltd., Männedorf, Switzerland) at excitation and emission wavelengths of 485 nm and 538 nm, respectively.

5. Western blot assay

Confluent HUVECs in 6 well or 6 cm dishes were pretreated with 2.5, 5, 10 μM of sulforaphene for 1 hour before stimulation with 10 ng/ml TNF-α. After different incubation times, HUVECs were washed with cold PBS and then harvested by using lysis buffer. Cell lysates were placed on ice for 30 minutes and then centrifugated at 14,000 rpm for 10 minutes. Equal amounts of protein samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking with 5% fat-free dry milk, the membranes were incubated with specific primary antibodies at 4°C overnight. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour, protein bands were detected using an enhanced chemiluminescence detection kit (GE Healthcare, London, UK).

6. Enzyme-linked immunosorbent assay

Levels of monocyte chemoattractant protein-1 (MCP-1) in culture supernatant were measured using Human MCP-1/CCL2 ELISA MAX Deluxe Sets (BioLegend, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, 100 μl of standards or culture supernatants were added to each well of a pre-coated plate with capture antibody and incubated for 2 hours at room temperature with shaking on a plate shaker. After 2 hours, each well was washed 4 times with wash buffer and added detection antibody and Avidin-HRP solution for 1 hour and 30 minutes, respectively. After washing the plate 5 times, 100 μl of TMB substrate solution was added and incubated for 20 minutes in dark. For stop reaction, 100 μl of stop solution was added and optical density of each well was determined using a microplate reader at wavelengths of 450 and 570 nm. The absorbance at 570 nm was subtracted from the absorbance at 450 nm. The standard curve was generated, and linear regression analysis was performed.

7. Real-time quantitative PCR

Total RNA was extracted from HUVECs using Trizol, RNA iso Plus (Takara Bio Inc., Shiga, Japan). RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was conducted with 1 μg/μl RNA using a PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio, Inc., Shiga, Japan). Primers of human VCAM-1 forward (5'-CCC TCC CAG GCA CAC ACA-3'), human VCAM-1 reverse (5'-GAT CAC GAC CAT CTT CCC AGG-3'), human MCP-1 forward (5'-TCG CCT CCA GCA TGA AAG TC-3'), human MCP-1 reverse (5'-GGC ATT GAT TGC ATC TGG CT-3'), human GAPDH forward (5'-CAG GGC TGC TTT TAA CTC TGG TAA A-3'), and human GAPDH reverse (5'-GGG

TGG AAT CAT ATT GGA ACA TGT AA-3') were purchased from Bioneer (Daejeon, Republic of Korea). For quantitative real-time PCR, iQ™ SYBR Green® Supermix and CFX Connect™ Realtime PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) were used. The expression level of target gene was calculated as a ratio of target gene relative to GAPDH in each sample.

8. Luciferase assay

The lentiviral expression vector pGF1-NF-κB-EF1-Puro (System Biosciences, Palo Alto, CA, 4USA) was transfected into HEK293T cells with the packaging vectors pMD2.G and psPAX2 (Addgene, Cambridge, MA, USA). JetPEI DNA transfection reagent (Polyplus-transfection, New York, NY, USA) was used for transfection. After the medium was harvested from HEK293T cells, HUVECs were transfected using the medium and 10 μg/ml polybrene (EMD Millipore, Billerica, MA, USA). Selected HUVECs by 1 μg/ml puromycin (InvivoGen, San Diego, CA, USA) were seeded at density of 1 x 10^4 cells/well in 96-well plates. HUVECs were pretreated with 2.5, 5, 10 μM of sulforaphene. After 1 hour, 10 ng/ml of TNF-α was added for stimulation. After incubation for 12 hours, cells were lysed with lysis buffer (0.1 M pH 7.8 PBS, 1% Triton X-100, 1 mM DTT and 2 mM EDTA) and luciferase activity was measured using a Luminoskan Ascent (Thermo Electron, Helsinki, Finland) according to the manufacturer's instructions.

9. Oil red O staining

RAW264.7 cells were seeded in 24-well plates at density of 3 x 10^5 cells/well and serum-starved with DMEM containing 1% (v/v) P/S. After 24 hours, the cells were pretreated with 2.5, 5 μM of sulforaphene for 1 hour and stimulated with 100 μg/ml of oxidized-LDL for foam cell formation and lipid accumulation. RAW264.7 cells were fixed with 4% formalin for 20 minutes and fixed cells were then stained with Oil red O solution dissolved in 60% isopropyl alcohol. After 20 minutes, the cells were washed with phosphate-buffered saline (PBS). Oil red O staining intensity of stained cells dissolved in isopropyl alcohol was measured at wavelength of 515 nm by using 96-well microplate reader.

10. The stability test of sulforaphene in water

The glass bottles of sulforaphene dissolved in water at the concentration of 10 μM were sealed. Samples were placed at -20, 4, 25℃ in constant temperature chambers and taken out after 7, 15, 30 days to measure the content of sulforaphene. The collected samples were filtered by 0.22 μm membrane filter and analyzed using reversed-phase Ultimate3000 HPLC (Thermo Dionex, USA). 0.3% (v/v) TFA in ultra-pure water and acetonitrile were used as the mobile phase system. The injection volume was 10 μl and the flow rate was 1 ml/min. The detection wavelength was 215 nm and the temperature of column oven was 30℃.

11. Statistical analysis

Statistical analyses were performed using SPSS (Statistical Analysis System Institute, 2017). All data were expressed as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference test. $P < 0.05$ was used for statistical significance.

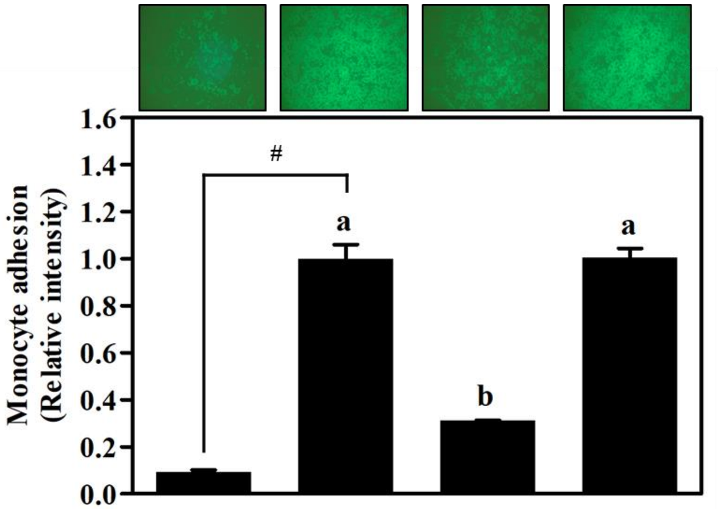
III. RESULTS

1. Radish (*Raphanus sativus*) seed extract effectively inhibits the adhesion of THP-1 monocytes to TNF-α-stimulated HUVECs compared to Ginkgo (*Ginkgo biloba*) leaf extract

To compare the preventive effects of radish seed extract and ginkgo leaf extract on monocyte-endothelial cell adhesion, HUVECs were pretreated with 80 μg/ml of radish seed extract and ginkgo leaf extract for 1 h and induced with 10 ng/ml of TNF-α. According to the results, Radish seed extract effectively inhibited THP-1 cell adhesion to HUVECs compared to the same concentration of ginkgo leaf extract (Fig. 1A). There is no cell cytotoxicity up to 80 μg/ml within 24 h (Fig. 1B).

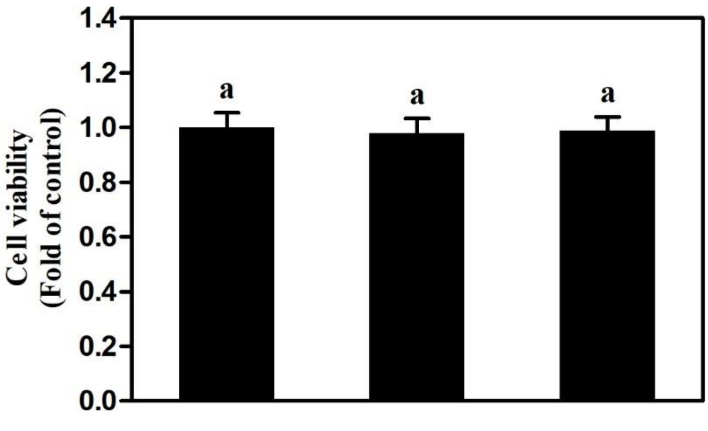
Figure 1

A



TNF-α (10 ng/ml)	-	+	+	+
Sample (80 μg/ml)	-	-	RSE	GLE

B



Sample (80 μg/ml)	-	RSE	GLE

*** RSE; Radish Seed Extract / GLE; Ginkgo Leaf Extract**

Figure 1. Inhibitory effect of Radish seed extract and Ginkgo leaf extract on monocyte-endothelial cell adhesion

A. Representative images of calcein-AM-labeled THP-1 cells adhering to HUVECs and THP-1 adhesion quantification of radish seed extract and ginkgo leaf extract was described in the Materials and Methods. **B.** Evaluation of cell cytotoxicity measured by MTT assay. Data are represented as means ± SD (n =3). Different letters in the bars indicate significant differences at $P < 0.05$.

2. Radish (*Raphanus sativus*) seed extract inhibits the adhesion of THP-1 monocytes to TNF-α-stimulated HUVECs

Preventive effect of radish seed extract on the adhesion of THP-1 monocytes to HUVECs was observed by using co-culture system of HUVECs and calcein-AM-labeled THP-1. HUVECs were pretreated with radish seed extract in indicated concentrations for 1 h and then stimulated with TNF-α for 5 h. Radish seed extract showed the inhibitory effect on binding of THP-1 monocytes to TNF-α-induced HUVECs (Fig. 2A). Moreover, there is no cytotoxic effect on cells up to 80 μg/ml within 24 h (Fig. 2B).

Figure 2

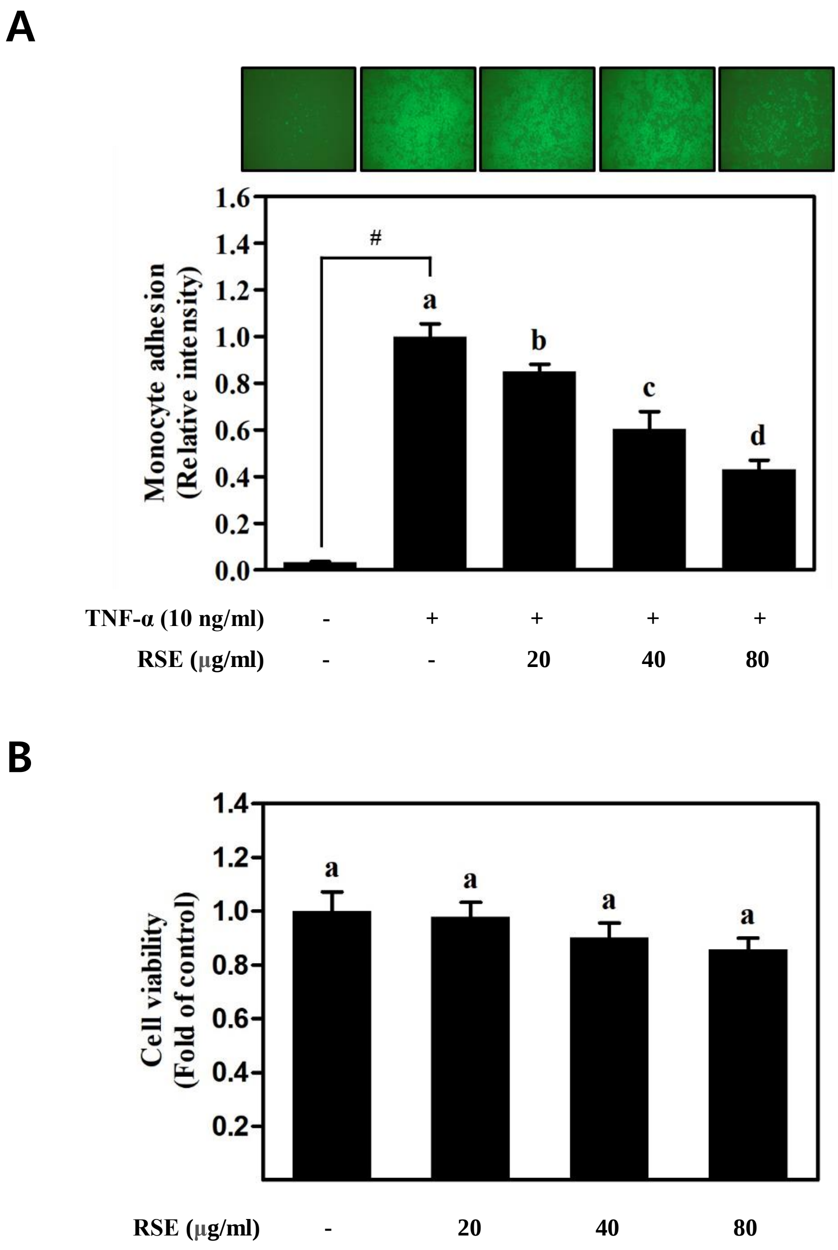

A
#
a
b
c
d
Monocyte adhesion (Relative intensity)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
TNF-α (10 ng/ml) - + + + +
RSE (μg/ml) - - 20 40 80
B
a
a
a
a
Cell viability (Fold of control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
RSE (μg/ml) - 20 40 80

Figure 2. Inhibitory effect of radish seed extract on monocyte-endothelial cell adhesion

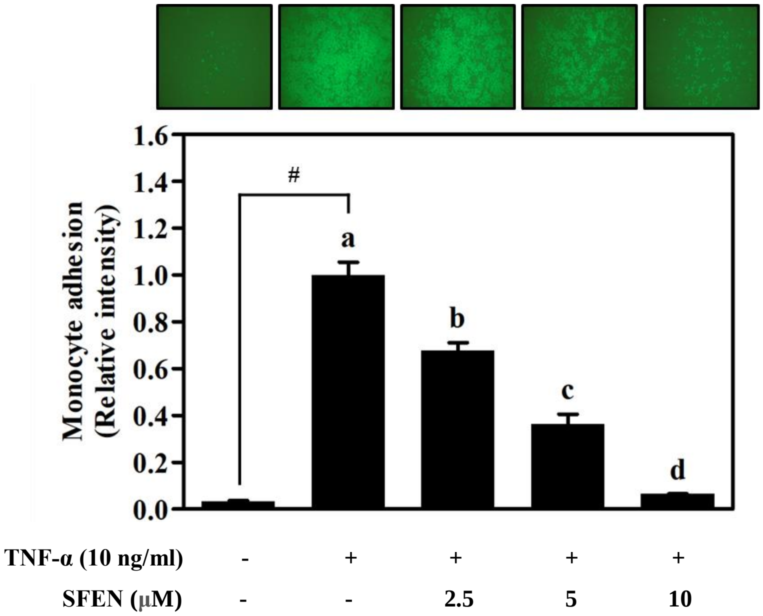
A. Representative images of calcein AM-labeled THP-1 cells adhering to HUVECs and quantification of THP-1 adhesion on HUVECs was described in the Materials and Methods. **B.** Evaluation of cell cytotoxicity measured by MTT assay. Data are represented as means ± SD (n =3). Different letters in the bars indicate significant differences at $P < 0.05$.

3. Sulforaphene attenuates the cell adhesion of THP-1 to TNF-α-stimulated HUVECs

Sulforaphene, an isothiocyanate, is an active component in radish seed. To identify whether sulforaphene exerts the preventive effect on THP-1 monocyte-endothelial adhesion, HUVECs were pretreated with 2.5, 5, 10 μM of sulforaphene for 1 h before TNF-α stimulation. With sulforaphene, THP-1 cell adhesion to HUVECs was significantly inhibited in a dose-dependent manner (Fig. 3A). In addition, there is no cytotoxic effect on cells up to 10 μM of sulforaphene within 24 h (Fig. 3B).

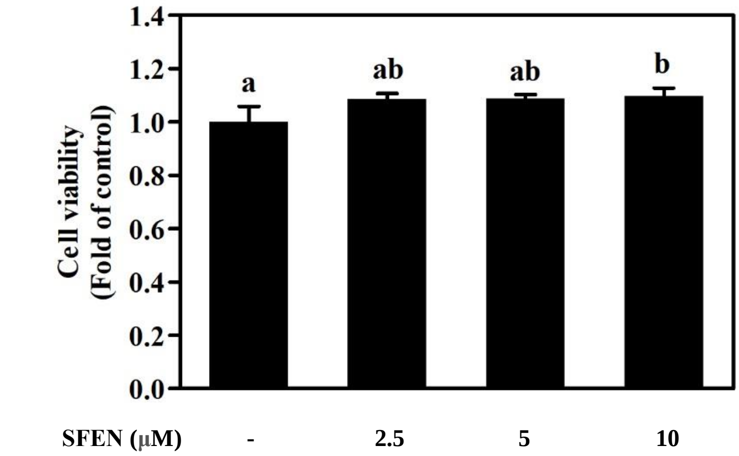
Figure 3

A



Monocyte adhesion
(Relative intensity)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
a
b
c
d
TNF-α (10 ng/ml) - + + + +
SFEN (μM) - - 2.5 5 10

B



Cell viability
(Fold of control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
ab
ab
b
SFEN (μM) - 2.5 5 10

Figure 3. Inhibitory effect of Sulforaphene on monocyte-endothelial cell adhesion

A. Representative images of calcein AM-labeled THP-1 cells adhering to HUVECs and quantification of THP-1 adhesion on HUVECs was described in the Materials and Methods. **B.** Evaluation of cell cytotoxicity measured by MTT assay. Data are represented as means ± SD (n =3). Different letters in the bars indicate significant differences at $P < 0.05$.

4. Sulforaphene suppresses VCAM-1 expression in TNF-α-stimulated HUVECs

VCAM-1 is a mediator that controls monocyte binding to endothelial cells[16]. To confirm the downregulation of VCAM-1 expression levels by sulforaphene, we performed Western blot analysis and RT-qPCR. The protein expression levels of VCAM-1 were reduced when TNF-α-induced HUVECs were pretreated with sulforaphene (Fig. 4A). Additionally, RT-qPCR results showed that sulforaphene attenuated VCAM-1 mRNA levels in a dose-dependent manner (Fig. 4B).

Figure 4

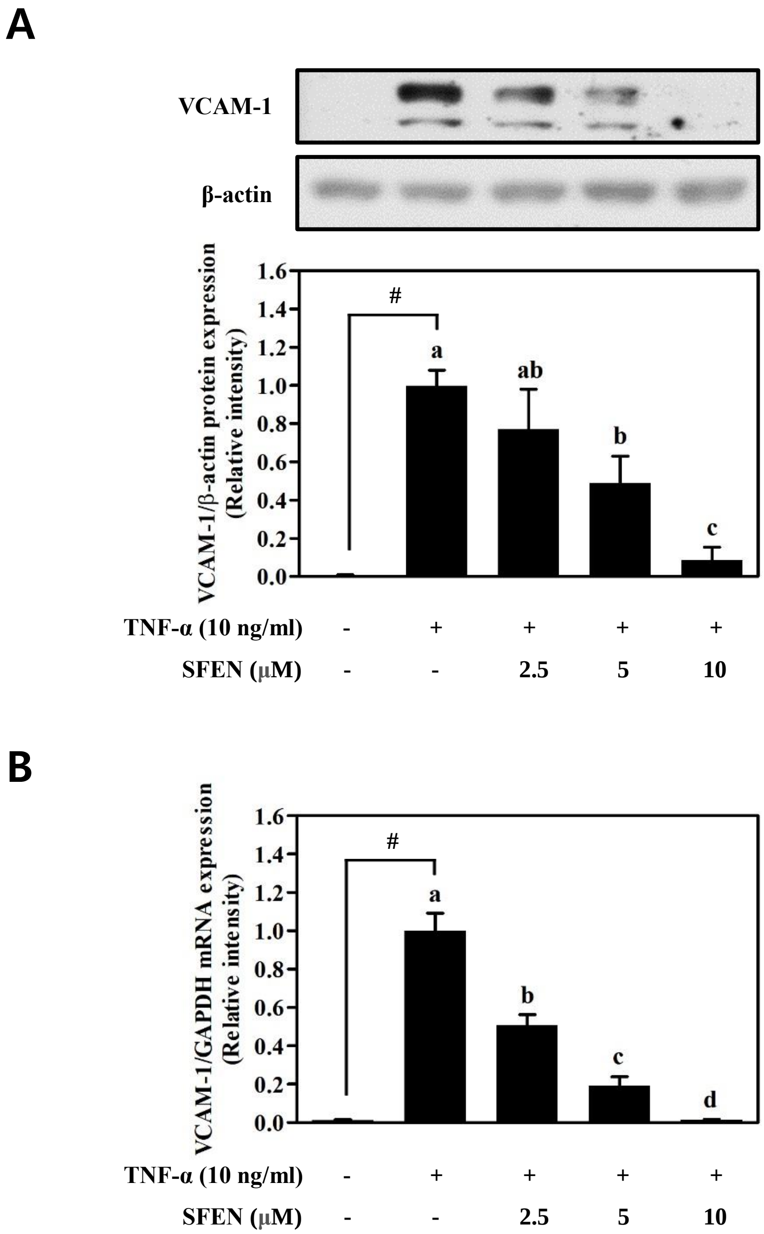

A
VCAM-1
β-actin
VCAM-1/β-actin protein expression (Relative intensity)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
a
ab
b
c
TNF-α (10 ng/ml) - + + + +
SFEN (μM) - - 2.5 5 10
B
VCAM-1/GAPDH mRNA expression (Relative intensity)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
a
b
c
d
TNF-α (10 ng/ml) - + + + +
SFEN (μM) - - 2.5 5 10

Figure 4. Effect of Sulforaphene on TNF-α-induced VCAM-1 expression in HUVECs

A. The levels of VCAM-1 protein expression in TNF-α-stimulated HUVECs measured by Western blot. **B.** The levels of VCAM-1 mRNA expression in TNF-α-stimulated HUVECs determined by RT-qPCR. All the data is represented as means ± SD (n =3). Different letters in the bars indicate significant differences at $P < 0.05$.

5. Sulforaphene suppresses MCP-1 expression in TNF-α-stimulated HUVECs

MCP-1 is a key chemokine that regulates the migration of monocytes into tissues[17]. To investigate the effect of sulforaphene on MCP-1 expression levels in TNF-α-stimulated HUVECs, we performed ELISA and RT-qPCR. ELISA results showed the protein expression levels of MCP-1 were downregulated with pretreatment of sulforaphene (Fig. 5A). In addition, RT-qPCR results showed that sulforaphene attenuated MCP-1 mRNA levels in a concentration-dependent manner (Fig. 5B).

Figure 5

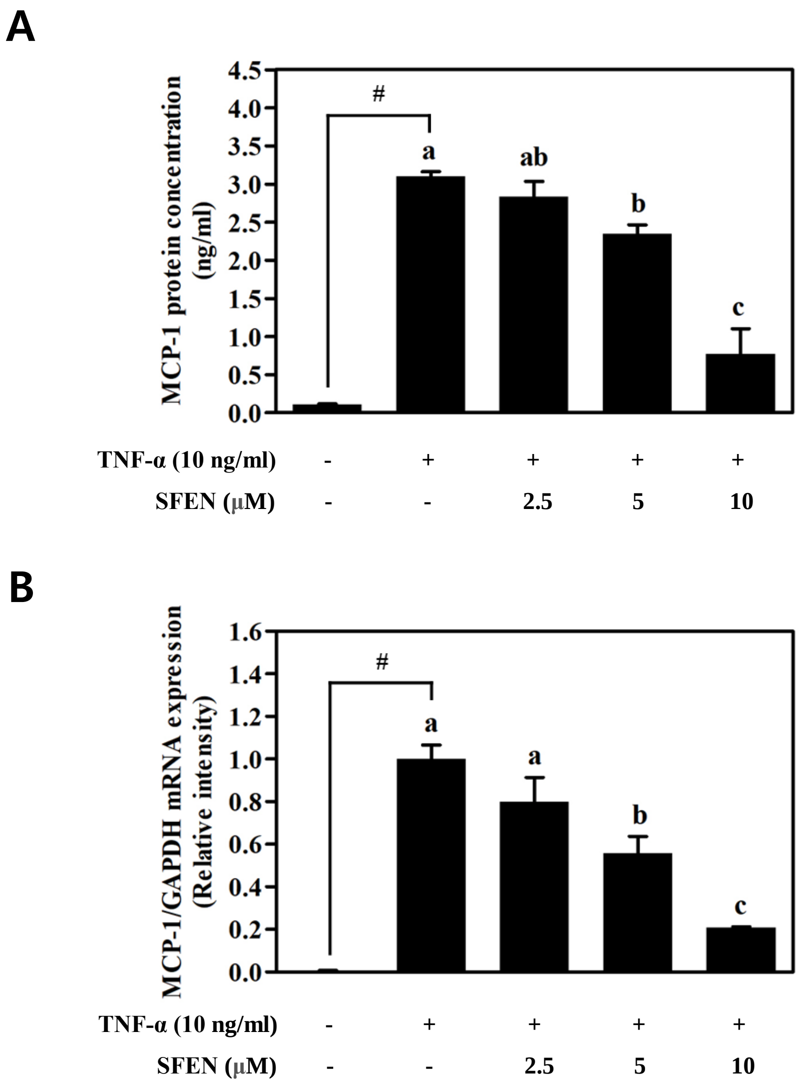

A
MCP-1 protein concentration (ng/ml)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
#
a
ab
b
c
TNF-α (10 ng/ml) - + + + +
SFEN (μM) - - 2.5 5 10
B
MCP-1/GAPDH mRNA expression (Relative intensity)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
a
a
b
c
TNF-α (10 ng/ml) - + + + +
SFEN (μM) - - 2.5 5 10

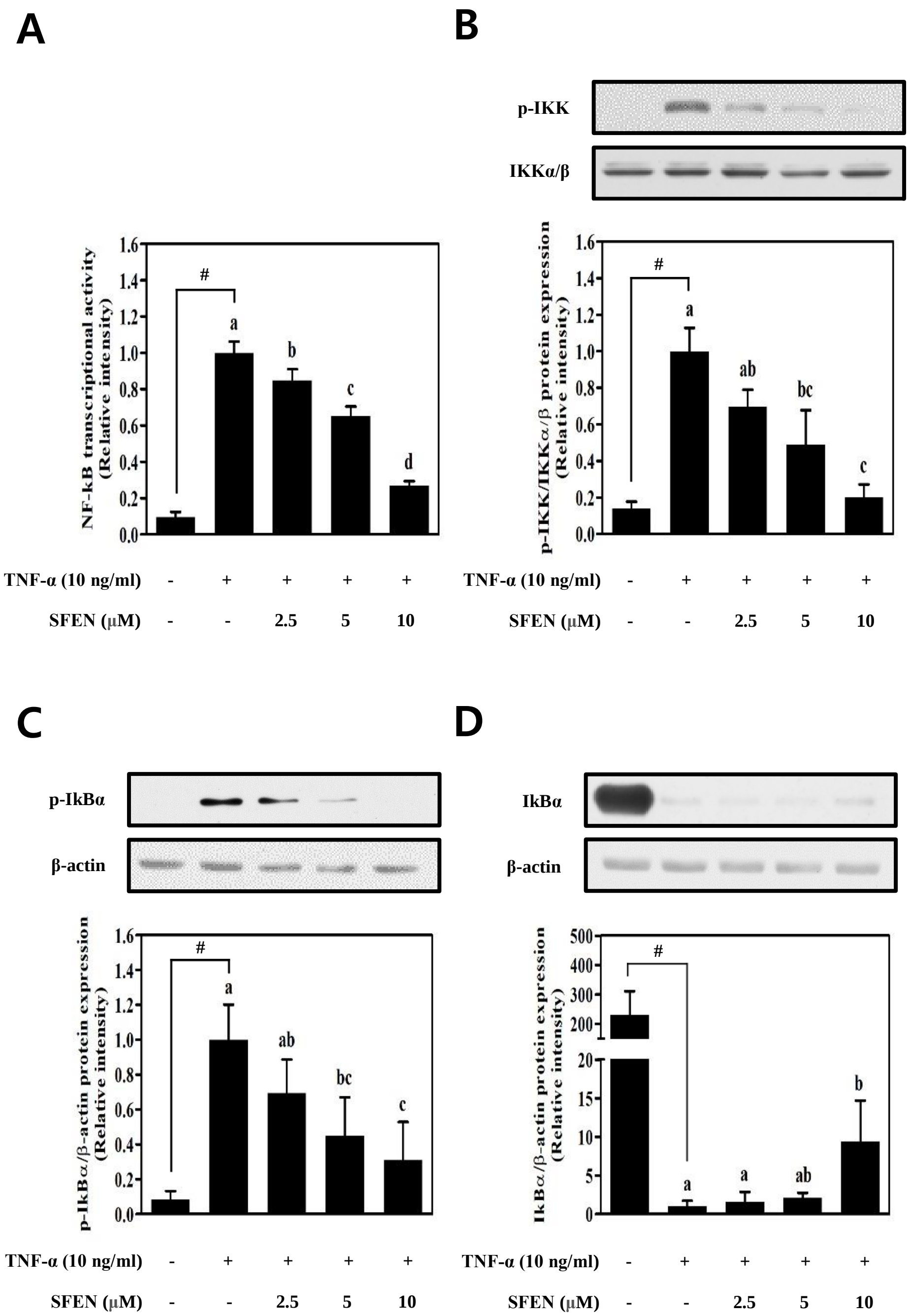
Figure 5. Effect of Sulforaphene on TNF-α-induced MCP-1 expression in HUVECs

A. The levels of MCP-1 protein expression in TNF-α-stimulated HUVECs measured by ELISA. **B.** The levels of MCP-1 mRNA expression in TNF-α-stimulated HUVECs measured by RT-qPCR. All the data is represented as means ± SD (n =3). Different letters in the bars indicate significant differences at $P < 0.05$.

6. Sulforaphene regulates NF-κB signaling pathway in TNF-α-stimulated HUVECs

NF-κB transcription factor is related to regulate VCAM-1 and MCP-1 expression in endothelial cells[18, 19]. We investigated the preventive effect of sulforaphene on NF-κB transcriptional activity in TNF-α-induced HUVECs by luciferase reporter gene assay. The results showed that sulforaphene downregulated NF-κB transactivation in a concentration-dependent manner (Fig. 6A). Also, using western blot analysis, we assessed the levels of phosphorylated IKK and IκBα, and total levels of IκBα protein. Western blot results showed that sulforaphene pretreatment inhibited phosphorylation of IKK (Fig. 6B) and IκBα (Fig. 6C) and degradation of IκBα (Fig. 6D), which are upstream factors of NF-κB.

Figure 6



A
B
p-IKK
IKKα/β
NF-kB transcriptional activity (Relative intensity)
p-IKK/IKKα/β protein expression (Relative intensity)
TNF-α (10 ng/ml) - + + + +
SFEN (μM) - - 2.5 5 10
C
D
p-IkBα
β-actin
IkBα
β-actin
p-IkBα/β-actin protein expression (Relative intensity)
IkBα/β-actin protein expression (Relative intensity)
TNF-α (10 ng/ml) - + + + +
SFEN (μM) - - 2.5 5 10

Figure 6. Effect of Sulforaphene on NF-κB signaling in TNF-α-stimulated HUVECs

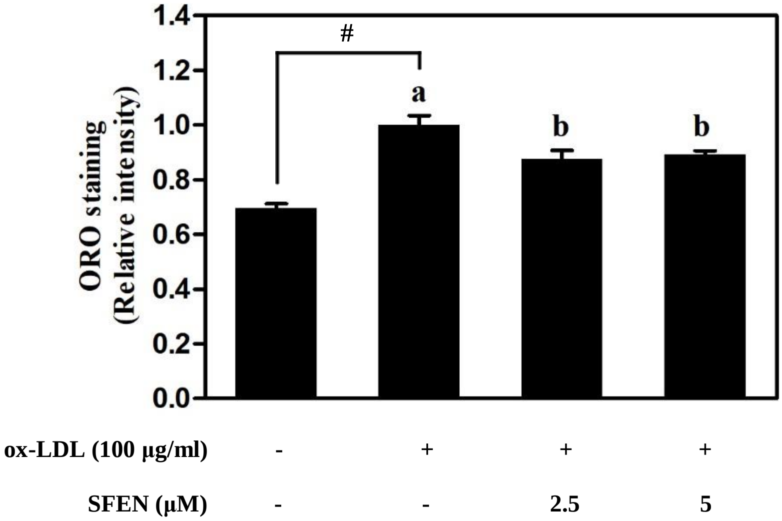
A. NF-κB activity in TNF-α-stimulated HUVECs determined by Luciferase assay. Western blot analysis for the levels of **B.** phosphorylated and total IKK protein expression, **C.** phosphorylated IκBα and **D.** total protein expression levels of IκBα. All the data is represented as means ± SD (n =3). Different letters in the bars indicate significant differences at $P < 0.05$.

7. Sulforaphene inhibits ox-LDL intake in RAW264.7 macrophages

After monocytes migrate into intima, monocytes differentiate into macrophage. Oxidized-LDL (ox-LDL) is accumulated into macrophage and it is related to formation of foam cells. To investigate the effect of sulforaphene on formation of foam cells in RAW264.7 macrophage, we performed Oil red O staining. Sulforaphene inhibited ox-LDL intake by macrophage and foam cell formation (Fig. 7A). Additionally, there is no cytotoxic effect on RAW264.7 cells up to 5 μM of sulforaphene within 24 h (Fig. 7B).

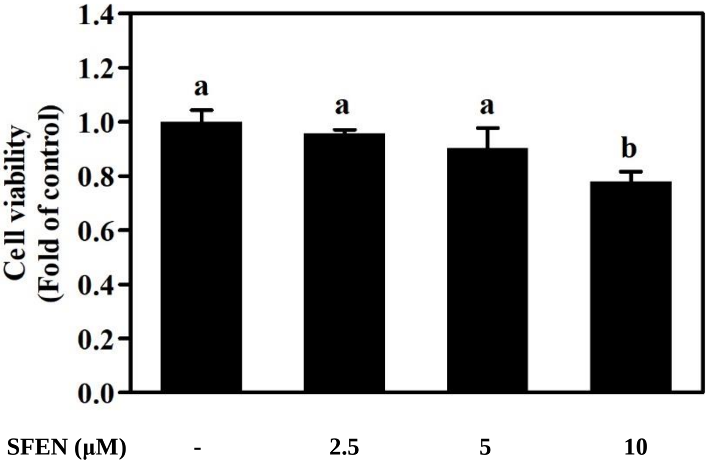
Figure 7

A



1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ORO staining
(Relative intensity)
#
a
b
b
ox-LDL (100 μg/ml) - + + +
SFEN (μM) - - 2.5 5

B



1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cell viability
(Fold of control)
a
a
a
b
SFEN (μM) - 2.5 5 10

Figure 7. Effect of Sulforaphene on ox-LDL accumulation in RAW264.7 cells

A. Oil red O staining intensity was measured at 515 nm to perform lipid accumulation in foam cells. **B.** In addition, there is no cytotoxic effect on cells up to 5 μM of sulforaphene within 24 h. Data are represented as means ± SD (n =3). Different letters in the bars indicate significant differences at $P < 0.05$.

Figure 8

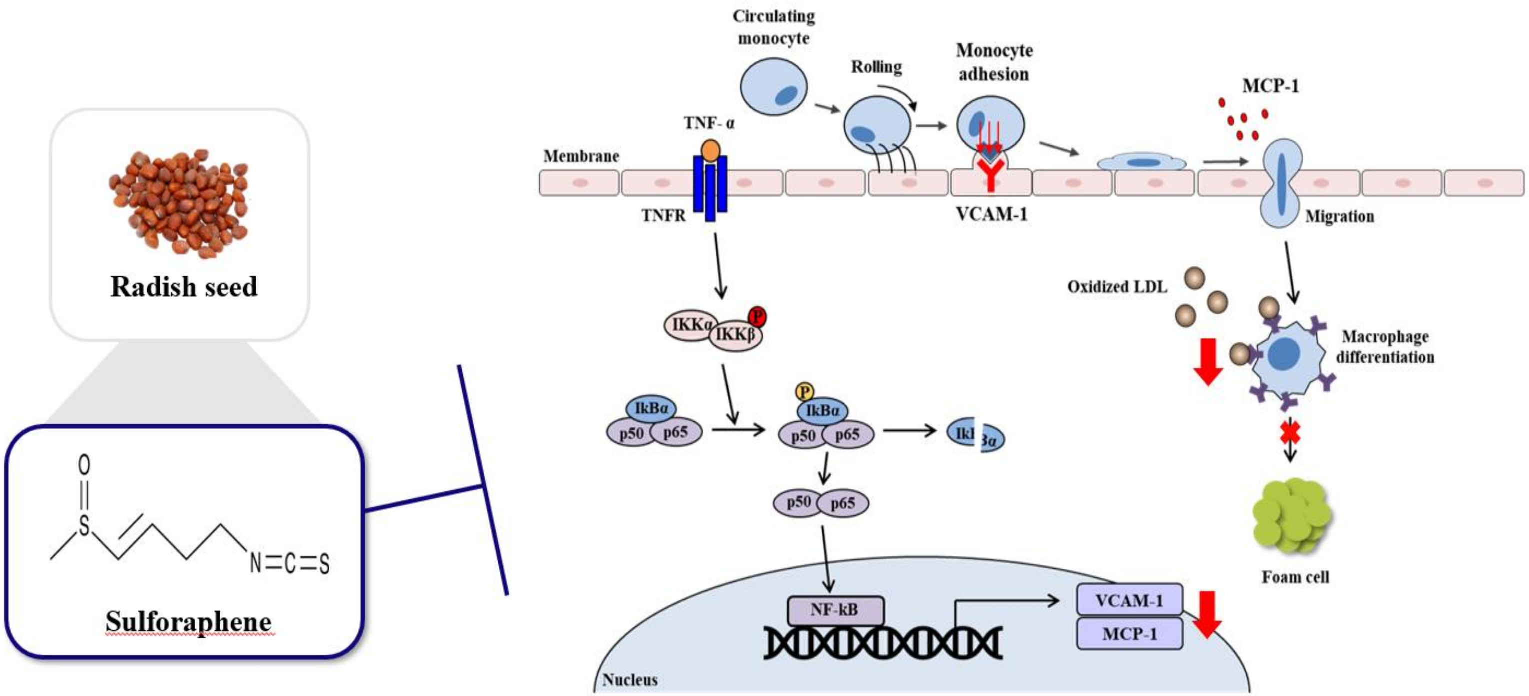


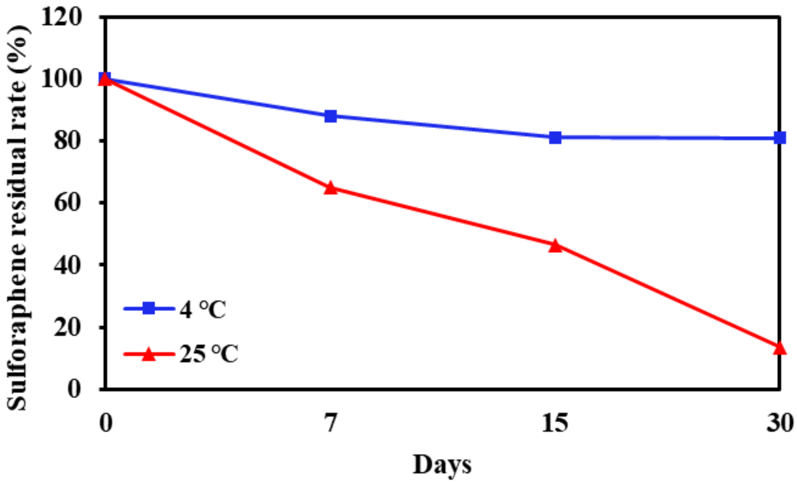
Figure 8. Proposed mechanism of Sulforaphene

9. Stability of sulforaphene decreases and new products are detected when sulforaphene is stored with water at 25℃

Next, we performed reversed-phase HPLC analysis to investigate whether sulforaphene is stable in the presence of water and at high temperature. The results showed the content of sulforaphene decreased at 4, 25℃ in a time-dependent manner and high temperature accelerated the sulforaphene degradation (Fig. 9A). When sulforaphene is stored at 25℃ in the presence of water, two new peaks were detected with larger molecular weights than sulforaphene and these products were expected to condensation products (Fig. 9B).

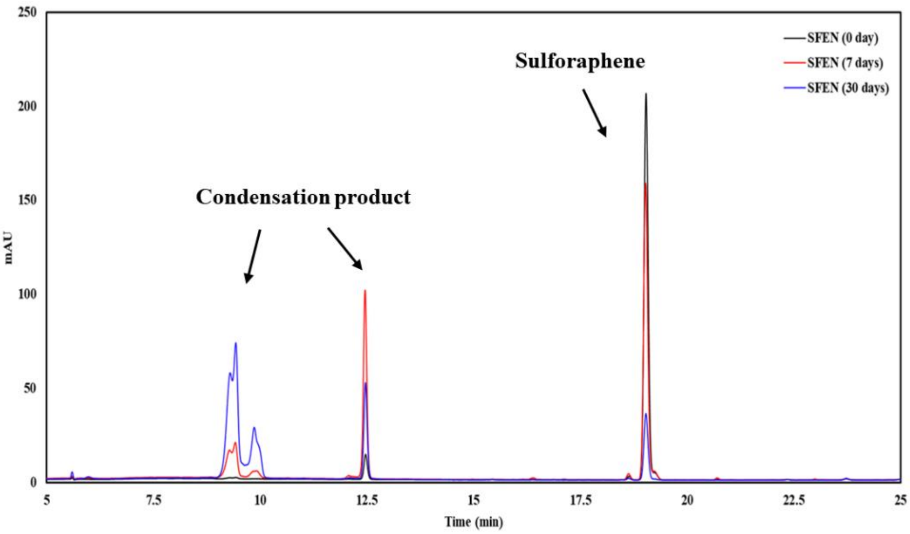
Figure 9

A



120
100
80
60
40
20
0
Sulforaphene residual rate (%)
4 °C
25 °C
0
7
15
30
Days

B



250
200
150
100
50
0
mAU
SFEN (0 day)
SFEN (7 days)
SFEN (30 days)
Sulforaphene
Condensation product
5
7.5
10
12.5
15
17.5
20
22.5
25
Time (min)

Figure 9. Effects of temperature and water content on the stability of sulforaphene

A. The residual rate of sulforaphene at 4, 25℃. **B.** Chromatogram of the reversed-phase HPLC analysis of sulforaphene and its condensation products.

10. Inhibitory effect on monocyte adhesion is reduced when sulforaphene is stored in aqueous medium at 25℃

It is important to determine the effects of high temperature and aqueous conditions during processing on the efficacy of sulforaphene. To compare the efficacy of sulforaphene stored at -20, 4, 25℃ for 7, 15, 30 days, we performed monocyte adhesion assay. Sulforaphene stored at -20℃ was used as a control. The result showed the inhibitory effect of sulforaphene on monocyte-endothelial cell adhesion was decreased in a time-dependent manner when sulforaphene was with water at the temperature of 25℃. It was due to the reduction of sulforaphene content and the production of two new products. Therefore, it indicated that the optimal temperature of sulforaphene was -20, 4℃.

Figure 10

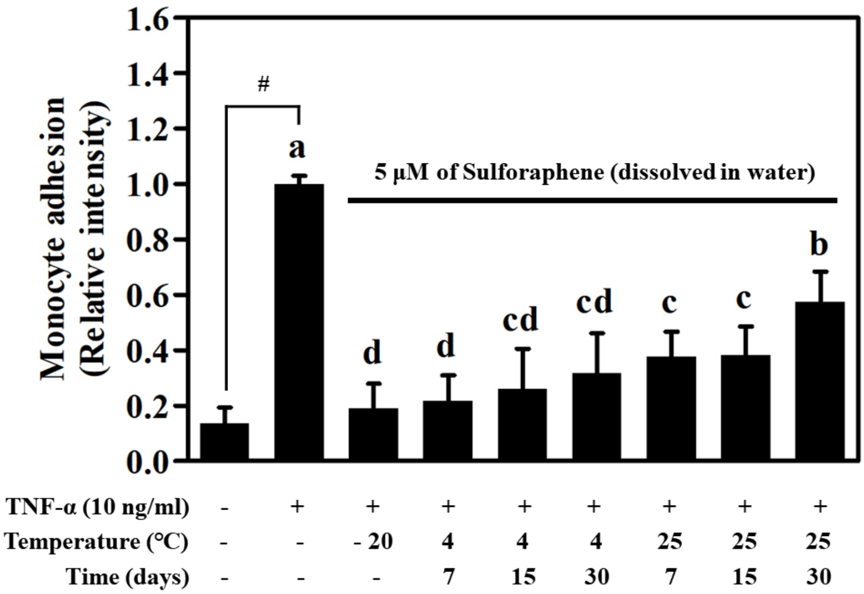


Figure 10. Effects of temperature and water content on the stability of sulforaphene

Quantification of THP-1 adhesion on HUVECs was described in the Materials and Methods. Data are represented as means ± SD (n =3). Different letters in the bars indicate significant differences at $P < 0.05$.

Ⅳ. DISCUSSION

Atherosclerosis is known as a chronic inflammatory disease[20]. Increased expression of adhesion molecules by vascular endothelial cells is considered to be one of the important causes of vascular inflammation. It causes monocyte recruitment to the atherosclerotic sites[21]. VCAM-1 is a cell adhesion molecule, expressed in vascular endothelial cells by the stimulation of inflammatory cytokines such as TNF-α and oxidized-LDL, and plays a role in firmly attaching monocytes to endothelial cells[22].

In this study, we investigated that radish seed extract and sulforaphene inhibited TNF-α-induced monocyte adhesion to vascular endothelial cells. Furthermore, we confirmed that sulforaphene, the main component of radish seeds, reduced TNF-α-induced VCAM-1 expression by using Western blot analysis and RT-qPCR. Inflammatory cytokines also increase the secretion of MCP-1, which induces the attraction of monocytes into the vascular intima[23]. We also found that sulforaphene inhibited the protein and mRNA expression of MCP-1. NF-κB is a transcription factor that activates the inflammatory response and regulates gene expression of cytokines and adhesion molecules[24]. IKK is an upstream kinase of IκBα in NF-κB pathway and it promotes phosphorylation of IκBα. This leads to polyubiquitination and proteasomal degradation. NF-κB dimer then translocates to the nucleus and binds to a specific gene promoter site. We confirmed that sulforaphene inhibited the activation of IKK and phosphorylation of IκBα, thereby reducing the activity of NF-κB transcription factor.

We also investigated whether sulforaphene inhibits foam cell formation. After monocytes enter into intima, it differentiates into macrophages and take up cholesterol[25]. Sulforaphene inhibited the accumulation of ox-LDL in macrophages at 2.5 to 5 μM. However, it is necessary to study the molecular mechanism how sulforaphene prevents ox-LDL-induced cholesterol accumulation in macrophages and foam cell formation. In sum, these results indicate that radish seed and sulforaphene inhibits monocyte-endothelial adhesion via NF-κB pathway and foam cell formation.

In this study, we also evaluated the stability of sulforaphene in aqueous solution at -20, 4, 25°C. Isothiocyanates such as sulforaphene are not stable in aqueous solution and at high temperature[26]. The isothiocyanate group has the electrophilic carbon atom which reacts with nucleophiles such as hydroxyl, thiol or amino groups and forms O-thiocarbamates, thiourea derivatives or dithiocarbamates[27]. Therefore, the stability of sulforaphene during storage is the major concern for its bioactivity[28]. We confirmed that new degradation products were formed when sulforaphene was stored in aqueous solution at 25°C. The inhibitory effect of sulforaphene on monocyte-endothelial adhesion is also reduced in that condition. It suggests that the degradation of sulforaphene may be due to the presence of water. It could be predicted that there is a close correlation between the water contents and the degradation of sulforaphene[29]. As a result, the optimal storage temperatures of sulforaphene in water -20, 4°C. These results suggested a good guideline for sulforaphene storage in industrial application. However, it remains to be determined what the degradation products are, analyzing the structure of the degradation products, and find out the cause of the diminished efficacy.

In conclusion, the present study indicated that radish seed extract and sulforaphene effectively inhibit monocyte adhesion to vascular endothelial cells. We also confirmed that these preventive effects of radish seed extract and sulforaphene are associated with the regulation of NF-κB mechanism, which is a transcriptional factor of VCAM-1 and MCP-1. And we found that sulforaphene was unstable in water and the optimal temperature for storage was -20°C, 4°C when it is stored in aqueous solution. However, *in vivo* and clinical studies are necessary to be applied to the functional foods industry.

Ⅴ. REFERENCES

1. Morris-Rosenfeld, S., M.J. Lipinski, and C.A. McNamara, Understanding the role of B cells in atherosclerosis: potential clinical implications. Expert Rev Clin Immunol, 2014. 10(1): p. 77-89.
2. Li, X., et al., The peptide lycosin-I attenuates TNF-alpha-induced inflammation in human umbilical vein endothelial cells via IkappaB/NF-kappaB signaling pathway. Inflamm Res, 2018. 67(5): p. 455-466.
3. Kablak-Ziembicka, A., et al., Carotid intima-media thickness, hs-CRP and TNF-alpha are independently associated with cardiovascular event risk in patients with atherosclerotic occlusive disease. Atherosclerosis, 2011. 214(1): p. 185-90.
4. Haddy, N., et al., IL-6, TNF-alpha and atherosclerosis risk indicators in a healthy family population: the STANISLAS cohort. Atherosclerosis, 2003. 170(2): p. 277-83.
5. Jia, Z., et al., Luteolin protects against vascular inflammation in mice and TNF-alpha-induced monocyte adhesion to endothelial cells via suppressing IKappaBalpha/NF-kappaB signaling pathway. J Nutr Biochem, 2015. 26(3): p. 293-302.
6. Zhang, T., et al., Atherosclerosis-Associated Endothelial Cell Apoptosis by MiR-429-Mediated Down Regulation of Bcl-2. Cell Physiol Biochem, 2015. 37(4): p. 1421-30.
7. Wu, G., et al., LincRNA-p21 regulates neointima formation, vascular smooth muscle cell proliferation, apoptosis, and atherosclerosis by enhancing p53 activity. Circulation, 2014. 130(17): p. 1452-1465.
8. Hamada, M., et al., MafB promotes atherosclerosis by inhibiting foam-cell apoptosis. Nat Commun, 2014. 5: p. 3147.
9. Kook, S.H., et al., Raphanus sativus L. seeds prevent LPS-stimulated inflammatory response through negative regulation of the p38 MAPK-NF-kappaB pathway. Int Immunopharmacol, 2014. 23(2): p. 726-34.

10. Kim, K.H., et al., 4-Methylthio-butanyl derivatives from the seeds of Raphanus sativus and their biological evaluation on anti-inflammatory and antitumor activities. J Ethnopharmacol, 2014. 151(1): p. 503-8.
11. Beevi, S.S., et al., Hexane extract of Raphanus sativus L. roots inhibits cell proliferation and induces apoptosis in human cancer cells by modulating genes related to apoptotic pathway. Plant Foods Hum Nutr, 2010. 65(3): p. 200-9.
12. Wu, X., Q.H. Zhou, and K. Xu, Are isothiocyanates potential anti-cancer drugs? Acta Pharmacol Sin, 2009. 30(5): p. 501-12.
13. Tian, G., et al., The mechanism of sulforaphene degradation to different water contents. Food Chem, 2016. 194: p. 1022-7.
14. Tian, G., et al., The stability and degradation mechanism of sulforaphene in solvents. Food Chem, 2016. 199: p. 301-6.
15. Tian, G., et al., The stability and degradation kinetics of Sulforaphene in microcapsules based on several biopolymers via spray drying. Carbohydr Polym, 2015. 122: p. 5-10.
16. Schnoor, M., et al., Crossing the Vascular Wall: Common and Unique Mechanisms Exploited by Different Leukocyte Subsets during Extravasation. Mediators Inflamm, 2015. 2015: p. 946509.
17. Gu, L., S.C. Tseng, and B.J. Rollins, Monocyte chemoattractant protein-1. Chem Immunol, 1999. 72: p. 7-29.
18. Adamson, P., M. Tighe, and J.D. Pearson, Protein tyrosine kinase inhibitors act downstream of IL-1 alpha and LPS stimulated MAP-kinase phosphorylation to inhibit expression of E-selectin on human umbilical vein endothelial cells. Cell Adhes Commun, 1996. 3(6): p. 511-25.
19. May, M.J., C.P. Wheeler-Jones, and J.D. Pearson, Effects of protein tyrosine kinase inhibitors on cytokine-induced adhesion molecule expression by human umbilical vein endothelial cells. Br J Pharmacol, 1996. 118(7): p. 1761-71.
20. Conti, P. and Y. Shaik-Dasthagirisaeb, Atherosclerosis: a chronic inflammatory disease mediated by mast cells. Cent Eur J Immunol, 2015. 40(3): p. 380-6.

21. Savoia, C., et al., Vascular inflammation and endothelial dysfunction in experimental hypertension. Int J Hypertens, 2011. 2011: p. 281240.
22. Singh, R.J., et al., Cytokine stimulated vascular cell adhesion molecule-1 (VCAM-1) ectodomain release is regulated by TIMP-3. Cardiovasc Res, 2005. 67(1): p. 39-49.
23. Hohensinner, P.J., et al., Monocyte chemoattractant protein (MCP-1) is expressed in human cardiac cells and is differentially regulated by inflammatory mediators and hypoxia. FEBS Lett, 2006. 580(14): p. 3532-8.
24. Liu, T., et al., NF-kappaB signaling in inflammation. Signal Transduct Target Ther, 2017. 2.
25. Jeong, S.J., M.N. Lee, and G.T. Oh, The Role of Macrophage Lipophagy in Reverse Cholesterol Transport. Endocrinol Metab (Seoul), 2017. 32(1): p. 41-46.
26. Kawakishi, S. and M. Namiki, Decomposition of Allyl Isothiocyanate in Aqueous Solution. Agricultural and Biological Chemistry, 2014. 33(3): p. 452-459.
27. Zhang, Y. and P. Talalay, Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms. Cancer Res, 1994. 54(7 Suppl): p. 1976s-1981s.
28. Kuang, P., et al., Separation and purification of sulforaphene from radish seeds using macroporous resin and preparative high-performance liquid chromatography. Food Chem, 2013. 136(2): p. 342-7.
29. Jin, Y., et al., Thermal degradation of sulforaphane in aqueous solution. J Agric Food Chem, 1999. 47(8): p. 3121-3.

국문 초록

내복자 추출물과 이에 함유된 설포라핀은 항염증, 항암 효능이 있다고 보고되었으나 이 소재의 심혈관질환 예방에 대한 연구는 거의 진행되지 않았다.

이에 본 연구에서는 내복자 추출물과 유효성분 설포라핀의 초기 동맥경화의 예방 효능을 관찰했으며 다음과 같은 결과를 얻었다.

내복자 추출물과 유효성분 설포라핀에 의해 단핵구의 혈관 내피세포 부착이 억제되는 것을 관찰하였다. 그리고 설포라핀이 TNF-α에 의해 증가된 혈관세포 부착분자 VCAM-1과 단핵구 이동에 관여하는 케모카인 MCP-1의 발현을 저해하는 것을 확인하였다. 또한 설포라핀이 전사조절 인자 NF-κB 신호 경로에 관여하는 IκB 인산화효소 (IKK)와 NF-κB 억제제 (IκB) α의 인산화를 농도의존적으로 저해하는 것을 확인하였다.

한편, 산화된 저밀도지단백 (ox-LDL)이 대식세포에 의해

섭취되면 거품세포가 형성되고 이것은 플라그 형성과 동맥경화에 영향을 미친다. 설포라펀이 대식세포의 지질 섭취 및 거품 세포 형성을 억제하는 것을 확인하였다. 결론적으로 설포라펀이 동맥경화 예방 소재로서 가능성이 있음을 확인하였다.

그러나, 설포라펀은 특정 용매와 높은 온도에서 불안정하다. 이에 본 연구에서는 7일, 15일, 30일 동안 수용액 조건과 -20℃, 4℃, 25℃ 온도 조건에서 설포라펀의 안정성을 확인하였다. 그 결과 설포라펀이 수용액 상태에서 저해되고 이량체로 예상되는 물질이 생성되며, 생성물의 경우 동맥경화 예방 효능이 감소하는 것을 확인하였다. 따라서 설포라펀 보관의 최적 온도는 -20℃, 4℃ 이며, 본 연구결과는 소재의 생산, 보관, 유통 측면에서 산업의 가이드라인 역할을 한다고 할 수 있다.

주요어: 설포라펀, 단핵구-혈관내피세포 부착, 동맥경화성 플라크, 동맥경화, 안정성;

학번: 2017-25119