A Thesis for the Degree of Master of Science

Gingerenone A, A Polyphenol Present in Ginger, Attenuates Monocyte-Endothelial Adhesion by Suppressing Phosphorylation of I Kappa B Kinase (IKK)

생강의 폴리페놀 성분인 진저레논 에이의 I Kappa B Kinase 인산화 조절을 통한 면역세포 부착 저해 기전 규명

August, 2015

By

Heejoo Kim

Department of Agricultural Biotechnology

Seoul National University

Abstract

In early stage of atherosclerosis, monocyte binds to endothelial layer, which causes inflammation in aorta. To prevent development of atherosclerosis, it is significant to inhibit the early pathogenesis. Ginger has been reported to have potent chemotherapeutic effects in cardiovascular diseases; however, many previous studies have focused on some specific compounds and mechanisms of action responsible remain unclear. Here, I analyzed major five ginger-derived compounds, and found that gingerenone A exhibited the strongest inhibitory effects against monocyte-endothelial adhesion. Furthermore, gingerenone A significantly suppressed expression of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1), key mediators in regulation of monocytic interaction to endothelial cells. Immunoblot analysis revealed that gingerenone A inhibited phosphorylation of I Kappa B Kinase (IKK) in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, a major pathway related to vascular inflammation and atherosclerosis as well. Taken together, the results of present study demonstrate that gingerenone

A attenuates monocyte adhesion and adhesion molecules expression in endothelial cells by suppressing phosphorylation of IKK.

Keywords: Gingerenone A; Gin A; Atherosclerosis; VCAM-1; MCP-1; Monocyte adhesion; Inflammation; IKK

Student ID: 2013-23880

Contents

Ⅰ. Introduction

Atherosclerosis is a chronic and progressive inflammatory disease of the cardiovascular system, and currently a major cause of death in the developed world through complications including coronary thrombosis, heart attack, hypertension and stroke [1]. Atherosclerosis is regarded as an intricate inflammatory disease with association of many different cell types. The atherosclerotic plaque formation is initiated by an accumulation of lipids in the artery wall, which trigger the excessive expression of various adhesion molecules, chemokines and cytokines by vascular endothelial cells [2]. Overexpressed adhesion molecules from endothelial cells is believed to play an important role in the atherosclerosis plaque formation by attracting monocytes to migrate into the sub-endothelial space [3]. Therefore, the inhibition or reversal of this endothelial activation constitutes a logical strategy for the prevention of atherosclerosis.

Vascular cell adhesion molecules-1 (VCAM-1) has been known to be overexpressed in atherosclerosis plaque and play pivotal roles in atherosclerosis plaque development by regulating vascular inflammation and monocyte adhesion [4, 5]. Also, Monocyte Chemotactic Protein-1 (MCP-1) has been widely known to recruit monocytes during atherogenesis [6]. Expression of these adhesion molecules is induced by pro-inflammatory stimulus such as tumor necrosis factor (TNF)-α or lipopolysaccharide (LPS) in endothelial cells [7, 8]. TNF-α is a significant cytokine in the inflammatory cascade occurred in primary atherosclerosis [9]. TNF receptor (TNFR) signaling which TNF-α induces in arterial cell walls causes pathogenesis of atherosclerosis by up-regulating expression of adhesion molecule and chemokine [10]. Toll-like receptor 4 (TLR4), the receptor for lipopolysaccharide (LPS), signaling pathway also leads to activation of transcription factor nuclear factor-κB (NF-κB) that up-regulates expression of adhesion molecule

and chemokine [11] and plays key role in the initiation and progression of atherosclerosis.

The VCAM-1 or MCP-1 promoter contains multiple binding sites for transcription factors, particularly nuclear factor-κB (NF-κB) [12]. NF-κB contributes regulation of VCAM-1 or MCP-1 gene expression in early pathogenesis of atherosclerosis [13, 14]. To regulate NF-κB activation in inflammation of atherosclerosis, it is important to control IκB and IKK protein [15].

It is well known that ginger has beneficial effects to alleviate pain and to interfere with the inflammatory cascade clinically as well as to be antioxidative, antitumorigenic and antimicrobial *in vitro* [16]. Also, Ginger has been reported to demonstrate potent chemopreventive and chemotherapeutic effects in cardiovascular diseases [17-19]. Ginger aqueous extract has a vasodilator effect by reducing blood pressure and anti-atherogenic effect by reducing low density lipoprotein (LDL)

cholesterol in plasma and inhibiting LDL oxidation [20, 21]. Major phenolic constituents in ginger to exert various effects are 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol [22]. However, although there are previous studies to research effects of ginger including ginger extract and ginger compounds in cardiovascular diseases, comparison with ginger components and potential underlying molecular mechanisms remains unknown.

In the present study, in order to find out a way to protect atherosclerosis, I compared five ginger derivatives to examine suppression of monocytic binding to endothelial cells, found that gingerenone A significantly inhibits monocyte adhesion and adhesion molecules, and examined gingerenone A has influence on effect of NF-κB action.

Ⅱ. Materials and methods

2.1. Chemicals

Gingerenone A was synthesized founded a previous report [23]. Synthesis of gingerenone A from curcumin was conducted by Dr. Thimmegowda. Curcumin, 6-shogaol, 6-gingerol, 8-gingerol, 10-gingerol, fetal bovine serum (FBS), medium 199, hydrocortisone, 2-mercaptoethanol, lipopolysaccharides (Escherichia coli O111:B4), the antibody against β-actin and calcein AM solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium were purchased from Welgene (Daegu, Korea). Fetal bovine serum (FBS), recombinant human epidermal growth factor, basic fibroblast growth factor and L-glutamine were purchased from Gibco (Grand Island, NY, USA). Recombinant human tumor necrosis factor-alpha was purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). Streptomycin/penicillin was purchased from Corning (Corning, NY,

USA). Antibodiy against VCAM-1 was purchased from Santa Cruz Biotechnology Incorporation (Santa Cruz, CA, USA). Phosphorylated IKKα/β, IKKα, IKKβ, phosphorylated IκBα and IκBα were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). 3-[dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from USB Corporation (Cleveland, OH, USA).

2.2. Cell culture

HUVECs derived from normal human tissue were obtained according to the principles outlined in the Declaration of Helsinki and supplied by Lonza (Walkersville, MD, USA). HUVECs were grown in monolayers in a 5% CO_2 incubator at 37℃ in Medium 199 media with HEPES containing 15% (v/v) FBS, 1 ng/ml EGF, 2 ng/ml bFGF, 1 ng/ml hydrocortisone, 2 mM L-glutamine and 1% (v/v) streptomycin/penicillin. HUVECs were used in all experiences between passages 6 and 12.

Human monocytic leukemia cell line THP-1 was purchased from Korean Cell Line Bank (KCLB) and cultured in RPMI 1640 media with 10% (v/v) FBS, 0.05 mM 2-mercaptoethanol, 1% (v/v) streptomycin/penicillin and incubation at 37°C (with humidity) in 5% CO_2.

2.3. Monocyte adhesion assay

To determine binding of monocyte to HUVECs, monocyte adhesion was assessed by monocyte adhesion assay as previously described [24]. The determination of monocyte adhesion to HUVECs was accomplished using THP-1 cells. In brief, HUVECs were cultured to confluence in 96-well plates and treated with 5, 10, 20 μM gingerenone A for 1 hour before the addition of 10 ng/ml human recombinant TNF-α (ProSpec) or 100 ng/ml LPS (Sigma-Aldrich). Cells were then incubated with medium containing TNF-α or LPS in the

continued presence or absence of gingerenone A for 6 hours. HUVECs were then removed and calcein AM labeled THP-1 cells with M199 medium were then added to HUVECs. After 1 hour incubation, HUVECs monolayer was washed with PBS to remove unbound monocytes. The adhered monocytes were determined by measuring the fluorescence using Infinite 200 PRO (Tecan group Ltd., Männedorf, Switzerland) at excitation and emission wavelengths of 485 nm and 538 nm.

2.4. Real-time quantitative PCR

Total RNA was extracted from cells and tissue using trizol, RNA iso Plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. For tissue, total RNA was recovered using an Ambion RNA Isolation Kit (Ambion Ltd., Huntingdon, Cambridgeshire, UK) according to the manufacturer's instructions. RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific,

Waltham, MA, USA). RNA (1 μg/μl) served as a template for the synthesis of cDNA using a PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio Incor.). cDNAs were amplified using the following primers (Bioneer, Daejeon, Korea) : 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 β-actin forward (5'- TCC TCA CCC TGA AGT ACC CCA T -3'); human β-actin reverse (5'- AGC CAC ACG CAG CTC ATT GTA -3'); human GAPDH forward (5'- CAG GGC TGC TTT TAA CTC TGG TAA A -3'); human GAPDH reverse (5'- GGG TGG AAT CAT ATT GGA ACA TGT AA -3'). For quantitative real-time PCR, the iQ™ SYBR Green® Supermix and CFX Connect™ Real-time PCR Detection System (Bio-Rad laboratories, Hercules, CA, USA) were used. By using the amplification program, the amount of target gene expression was calculated as a ratio of a target transcript

relative to β-actin and CD31 in the each samples.

2.5. Cell viability assay

In order to assess cell viability, HUVECs were seeded into 96-well plates. After 24 hours, HUVECs were treated with each concentration of gingerenone A from 0 to 40 μM and incubated for 24 hours. MTT solution was added to each well to be 0.5 mg/ml concentration and the cells were incubated for 1 hour. The dark formazan crystals that were formed by the intact cells were dissolve in dimethyl sulfoxide, and the absorbance at 570 nm was measured with a microplate reader. The results are expressed as percent MTT reduction relative to the absorbance of the untreated cells.

2.6. Western blot assay

HUVECs were grown to confluence in 6 cm dishes and treated with 5, 10, 20 μM gingerenone A for 1 hour before the addition of 10 ng/ml human recombinant TNF-α (ProSpec) or 100 ng/ml LPS (Sigma-Aldrich). Cells were then incubated with medium containing TNF-α or LPS in the continued presence or absence of gingerenone A for 6 hours. HUVECs were washed with cold PBS and harvested by scraping into ice-cold RIPA buffer (Cell Signaling Biotechnology). The extracts were incubated in ice over 30 minutes and centrifuged at 14,000 rpm for 10 minutes. Protein level was determined using Protein Assay Reagent (Bio-Rad). Protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane (Emdmillipore, Billerica, MA, USA). Membranes were blocked in 5% fat-free dry milk and then incubated with a specific primary antibody at 4℃ overnight. After

incubation with horseradish peroxidase-conjugated secondary antibodies, protein bands were detected using an enhanced chemiluminescence detection kit (GE Healthcare, London, UK).

2.7. The enzyme-linked immunosorbent assay

The levels of MCP-1 in culture supernatant were determined by Human MCP-1/CCL2 ELISA MAX Deluxe Sets (BioLegend, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, 100 μl of standard cytokine or culture supernatant was added to each well and incubated for 2 hours at room temperature. Each well was washed four times with wash buffer, incubated for 1 hour with detection antibody, incubated for 30 minutes with Avidin-HRP solution and then for 20 minutes with substrate solution. The optical density of each well was determined using a microplate reader at 450 nm and 570 nm. The absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

A standard curve for cytokine was generated. On the basis of this standard curve, linear regression analysis was performed.

2.8. Statistical analysis

Statistical analyses were performed using SPSS statistics (SPSS Inc., Chicago, IL, USA). Data are expressed as mean±standard error of the mean (SEM), and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test. A probability value of $p < 0.05$ was used as the criterion for statistical significance.

Ⅲ. Results

3.1. Gingerenone A has the most suppressive effects on monocyte-endothelial adhesion and expression of adhesion molecule among ginger derivatives

To compare the effects of ginger compounds on binding of monocytes to endothelial cells, HUVECs were treated with gingerenone A, 6-shogaol, 6-gingerol, 8-gingerol, 10-gingerol and curcumin as a positive control which is well known to be potent compound for preventing inflammation [25]. Gingerenone A intensely suppressed adhesion of monocytes to endothelial cells among other compounds (Fig. 1A and 1B). Also, gingerenone A inhibited VCAM-1 mRNA expression exceedingly in HUVECs treated with 5 μM of gingerenone A, curcumin, 6-shogaol and 6-gingerol (Fig. 1C). The concentrations at which gingerenone A exhibited inhibition effects were not cytotoxic towards (Fig. 1D). These results suggest that gingerenone A has the most

protective effects on adherence of monocytes to endothelial cells and suppressive effects on expression of adhesion molecule among ginger derivatives.

Figure 1

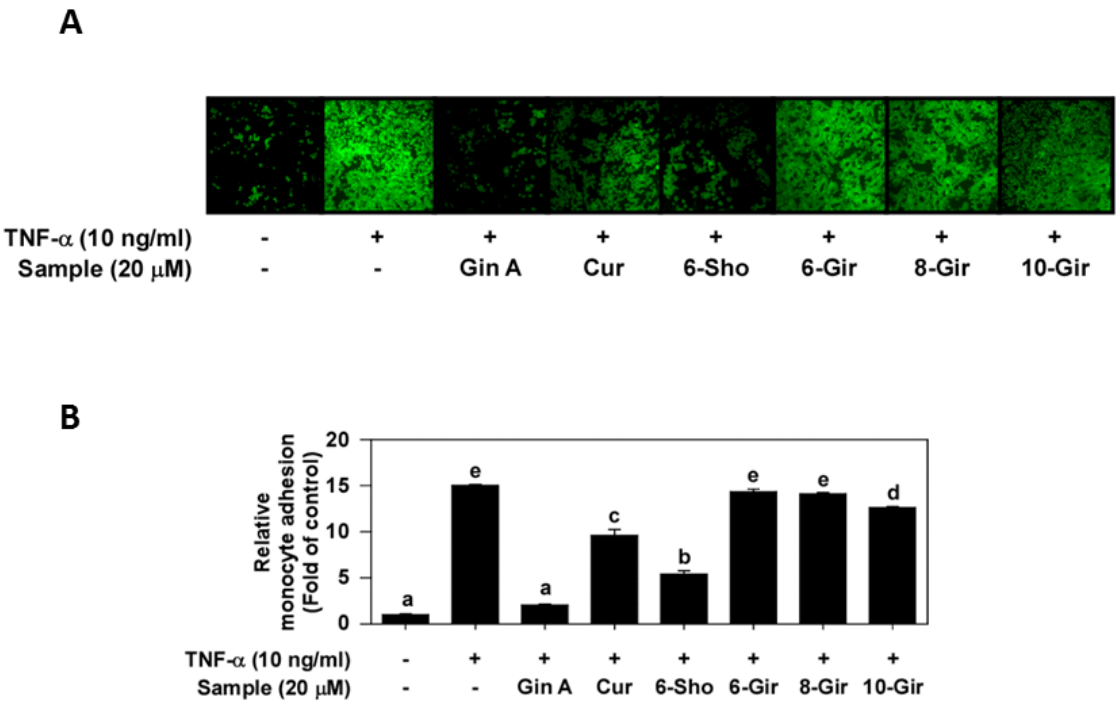
A
TNF-α (10 ng/ml) - + + + + + + +
Sample (20 μM) - - Gin A Cur 6-Sho 6-Gir 8-Gir 10-Gir
B
Relative monocyte adhesion (Fold of control)
20
15
10
5
0
a e a c b e e d
TNF-α (10 ng/ml) - + + + + + + +
Sample (20 μM) - - Gin A Cur 6-Sho 6-Gir 8-Gir 10-Gir
C
VCAM-1
Relative mRNA expression (Fold of control)
400
300
200
100
0
a d b c c e
TNF-α (10 ng/ml) - + + + + +
Sample (5 μM) - - Gin A Cur 6-Sho 6-Gir
D
Relative cell viability (% of control)
120
80
40
0
Gin A (μM) - 5 10 20 40

Figure 1. Effects of ginger compounds on monocyte-endothelial adhesion

(A-B) After pre-treating the HUVECs with each ginger compounds for 1 h, the cells were stimulated with TNF-α, then Calcein-AM labeled THP-1 cells were mixed with HUVECs. (A) Representing images of THP-1 and HUVEC binding. (B) Quantification of the number of THP-1 bound with HUVEC was conducted as described in Material and Method. (C) HUVECs were pre-treated with each ginger compounds for 1 h before the addition of TNF-α. The levels of mRNA expression were determined by real-time quantitative PCR as described in Materials and Methods. (D) Cytotoxicity test with gingerenone A. HUVECs were treated with gingerenone A for 24 h. Data are represented as mean ± SEM values of at least three independent experiments. Means with letters (a–d) within a graph are significantly different from each other at $p < 0.05$.

3.2. Gingerenone A inhibits interaction of monocytes to endothelial cells

To investigate effects of gingerenone A on monocyte-endothelial adhesion, I examined binding of monocytes in HUVECs induced by two different vascular inflammatory factors, TNF-α or LPS, respectively. Gingerenone A significantly suppressed binding of monocytes in TNF-α stimulated HUVECs in a dose-dependent manner (Fig. 2A and 2B). In addition, gingerenone A also attenuated interaction of monocytes in HUVECs stimulated by LPS (Fig. 2C and 2D). These results indicate that gingerenone A has inhibitory effects on adherence of monocytes to endothelial cells.

Figure 2

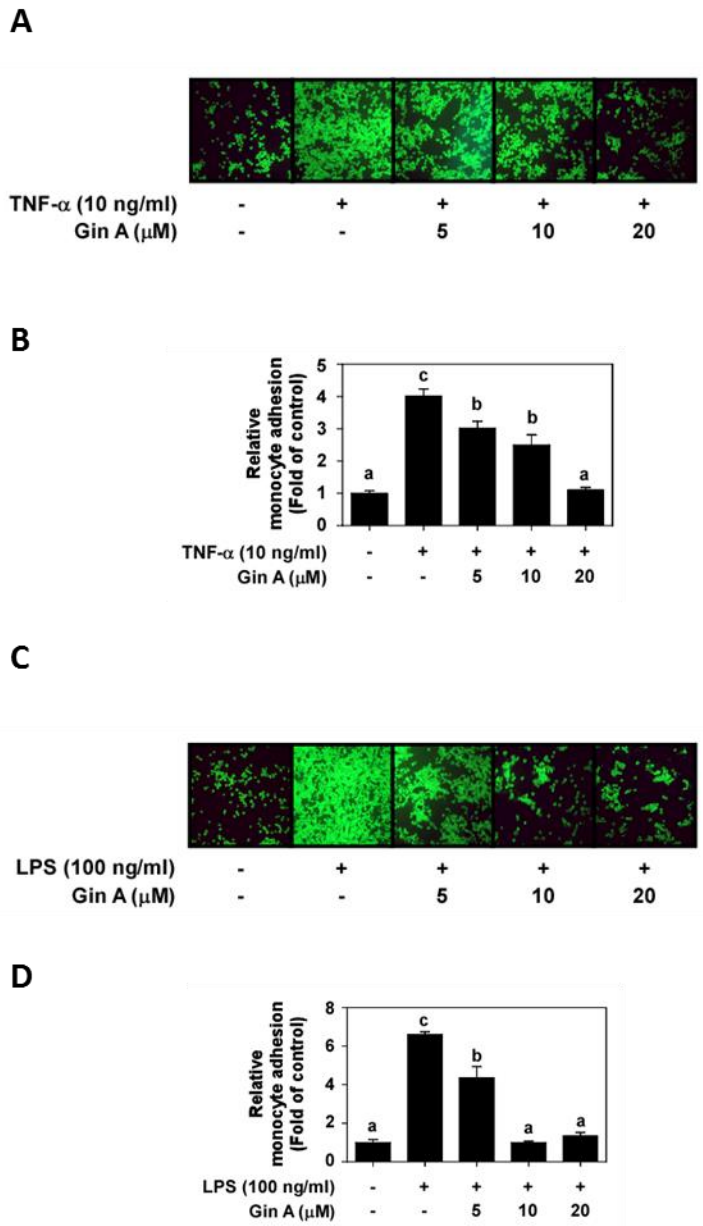
A
TNF-α (10 ng/ml) - + + + +
Gin A (μM) - - 5 10 20
B
Relative monocyte adhesion (Fold of control)
0 1 2 3 4 5
a c b b a
TNF-α (10 ng/ml) - + + + +
Gin A (μM) - - 5 10 20
C
LPS (100 ng/ml) - + + + +
Gin A (μM) - - 5 10 20
D
Relative monocyte adhesion (Fold of control)
0 2 4 6 8
a c b a a
LPS (100 ng/ml) - + + + +
Gin A (μM) - - 5 10 20

Figure 2. Effects of gingerenone A on monocyte-endothelial adhesion

(A-D) After pre-treating the HUVECs with gingerenone A for 1 h, the cells were stimulated with TNF-α or LPS, then Calcein-AM labeled THP-1 cells were mixed with HUVECs. (A, C) Representing images of THP-1 and HUVEC binding. (B, D) Quantification of the number of THP-1 bound with HUVEC was conducted as described in Material and Method. Data are represented as mean ± SEM values of at least three independent experiments. Means with letters (a–d) within a graph are significantly different from each other at $p < 0.05$.

3.3. Gingerenone A suppresses TNF-α or LPS induced VCAM-1 expression in HUVECs

When monocytes in blood adhere to endothelial cells at sites of inflammation, VCAM-1 is one of the most important molecules to mediate the adherence [26]. To identify regulatory effects of gingerenone A on VCAM-1 expression, I assessed the effects of gingerenone A on TNF-α or LPS induced protein and mRNA expression of VCAM-1 in HUVECs. Gingerenone A treatment decreased TNF-α induced protein level of VCAM-1 (Fig. 3A) as well as mRNA level (Fig. 3B) in HUVECs. As similar results, gingerenone A treatment also decreased LPS induced protein and mRNA levels of VCAM-1 in HUVECs (Fig. 3C and 3D). These data suggest that gingerenone A inhibit protein and mRNA expression of VCAM-1 in endothelial cells.

Figure 3

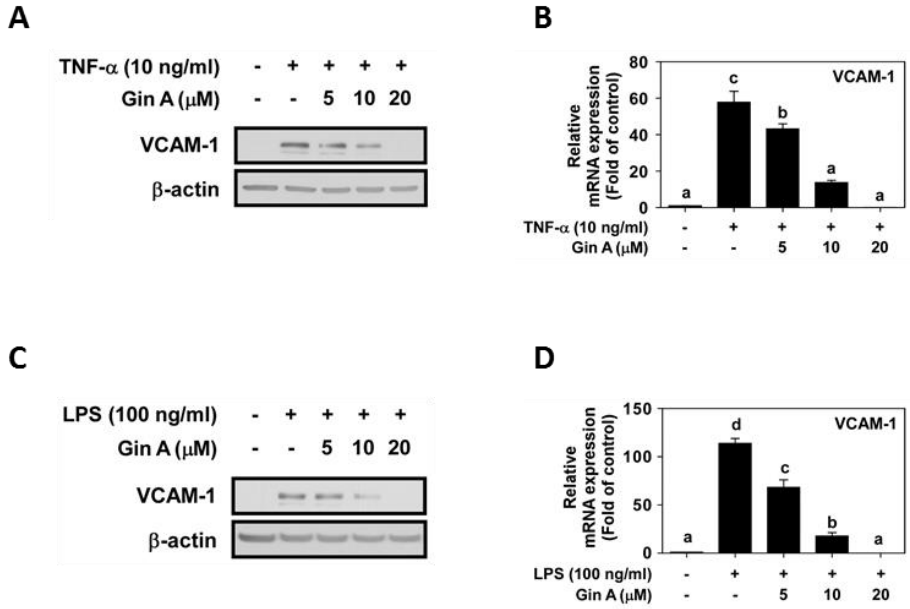
A
TNF-α (10 ng/ml) - + + + +
Gin A (μM) - - 5 10 20
VCAM-1
β-actin
B
VCAM-1
Relative mRNA expression (Fold of control)
80
60
40
20
0
a
c
b
a
a
TNF-α (10 ng/ml) - + + + +
Gin A (μM) - - 5 10 20
C
LPS (100 ng/ml) - + + + +
Gin A (μM) - - 5 10 20
VCAM-1
β-actin
D
VCAM-1
Relative mRNA expression (Fold of control)
150
100
50
0
a
d
c
b
a
LPS (100 ng/ml) - + + + +
Gin A (μM) - - 5 10 20

Figure 3. Effects of gingerenone A on VCAM-1 expression in HUVECs

(A-D) After pre-treating the HUVECs with gingerenone A for 1 h, the cells were stimulated with TNF-α or LPS. (A, C) Representative western blot gel analysis of VCAM-1 protein expression. (B, D) The levels of VCAM-1 mRNA expression were determined by real-time quantitative PCR as described in Materials and Methods. Data are represented as mean ± SEM values of at least three independent experiments. Means with letters (a–d) within a graph are significantly different from each other at $p < 0.05$.

3.4. Gingerenone A attenuates MCP-1 expression at protein and mRNA levels in TNF-α or LPS induced HUVECs

Contribution of inflamed MCP-1 expression is potent in endothelium since role of MCP-1 is regulating adherence of monocytes to endothelial cells and recruiting more monocytes [27]. To elucidate inhibitory effects of gingerenone A on MCP-1 expression, another important chemotactic factor for monocyte, I assessed the effects of gingerenone A on expression of MCP-1 in TNF-α or LPS stimulated HUVECs. Gingerenone A treatment reduced TNF-α induced MCP-1 protein expression (Fig. 4A) as well as mRNA expression (Fig. 4B) in HUVECs. Similarly, gingerenone A treatment decreased both protein and mRNA levels of MCP-1 in LPS-stimulated HUVECs (Fig. 4C and 4D). These results suggest that gingerenone A suppress protein and mRNA expression of MCP-1 in endothelial cells.

Figure 4

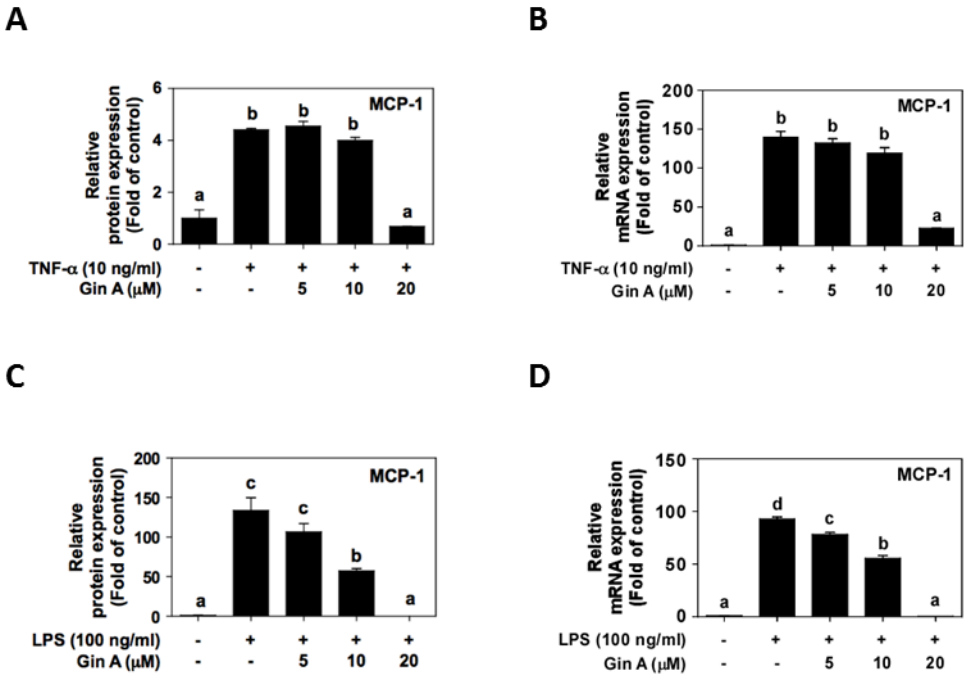
A
B
C
D
MCP-1
Relative protein expression (Fold of control)
Relative mRNA expression (Fold of control)
TNF-α (10 ng/ml)
LPS (100 ng/ml)
Gin A (μM)
- + + + +
- - 5 10 20
a b b b a
a c c b a
a b b b a
a d c b a

Figure 4. Effects of gingerenone A on MCP-1 expression in HUVECs

(A-D) After pre-treating the HUVECs with gingerenone A for 1 h, the cells were stimulated with TNF-α or LPS. (A, C) Representative ELISA analysis of MCP-1 protein expression. (B, D) The levels of MCP-1 mRNA expression were determined by real-time quantitative PCR as described in Materials and Methods. Data are represented as mean ± SEM values of at least three independent experiments. Means with letters (a–d) within a graph are significantly different from each other at $p < 0.05$.

3.5. Gingerenone A suppresses phosphorylation of proteins involved in NF-κB signaling in HUVECs stimulated by TNF-α or LPS

Since NF-κB signaling pathway is well known to regulate inflammatory response happened in early stage of atherosclerosis, I checked phosphorylation of IKK and IκBα which are key regulators in NF-κB signaling pathway. Gingerenone A treatment down-regulated TNF-α induced phosphorylation of IKK in concentration-dependent manner as well as IκBα, its downstream signaling protein (Fig. 5A). Gingerenone A treatment in LPS also induced HUVECs dose dependently reduced phosphorylation of IKK and IκBα (Fig. 5B). I also found that level of basal IκBα is lowered in TNF-α or LPS induced HUVECs and gingerenone A treatment recover the basal level of IκBα. (Fig. 5A and 5B). These data indicate that gingerenone A attenuates NF-κB action in endothelial cells.

Figure 5

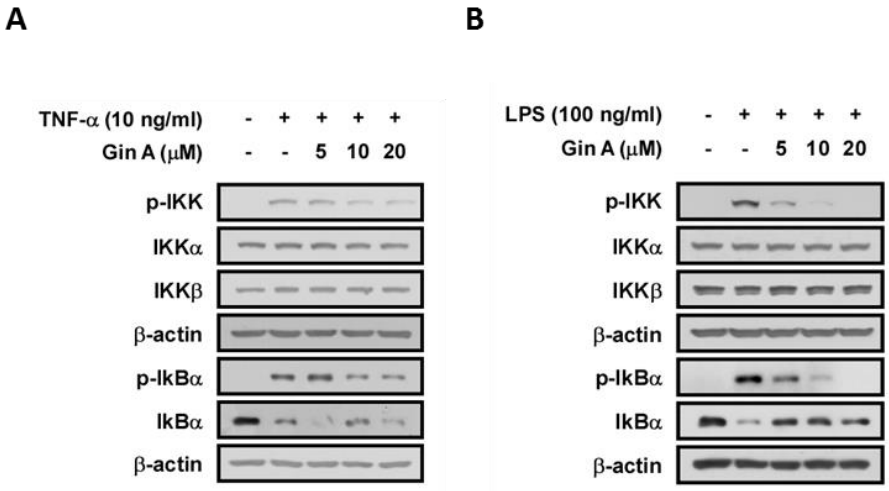
A
TNF-α (10 ng/ml) - + + + +
Gin A (μM) - - 5 10 20
p-IKK
IKKα
IKKβ
β-actin
p-IkBα
IkBα
β-actin
B
LPS (100 ng/ml) - + + + +
Gin A (μM) - - 5 10 20
p-IKK
IKKα
IKKβ
β-actin
p-IkBα
IkBα
β-actin

Figure 5. Effects of gingerenone A on NF-κB signaling in HUVECs

(A) Immunoblot analysis of the levels of phosphorylated and total protein. After pre-treating the HUVECs with gingerenone A for 1 h, the cells were stimulated with TNF-α for 5 min (IκBα) or for 15 min (IKK). (B) Immunoblot analysis of the levels of phosphorylated and total protein. After pre-treating the HUVECs with gingerenone A for 1 h, the cells were stimulated with LPS for 60 min (IκBα and IKK). Data are represented as mean ± SEM values of at least three independent experiments. Means with letters (a–d) within a graph are significantly different from each other at $p < 0.05$.

Ⅳ. Discussion

It is well known that atherosclerosis accounts for big portion in cardiovascular disease which is one of major causes of death. Also, it is mentioned that atherosclerosis is the primary cause of stroke and other heart diseases [28, 29]. Atherosclerosis development is divided into early stage and late stage [30]. In early stage, lipid accumulate, monocytes adhere to endothelial cells and inflammation happen. Then, in late stage of atherosclerosis, smooth muscle cells proliferate and migrate, which leads to formation of plaque and thrombosis so, surgical operation is needed in this stage [31]. Accordingly, it is significant to regulate the progress in early stage of atherosclerosis in order to prevent the disease.

However, almost products are extracts from food sources only for blood circulation in market of dietary supplement. Unfortunately, dietary supplements have side effects to increase of bleeding risks. Since phytochemicals have benefits to have protective effects and to be safe to

eat for whole life, it is needed to develop novel phytochemical which has protective effects on inflammation. Therefore, substances which are safe, effective and useful in early stage of atherosclerosis should be studied. In this study, I focused on anti-inflammatory effects related to early pathogenesis of atherosclerosis in ginger active compounds. Although effects to reduce lipid level or regulate inflammation in ginger are well studied [22, 32], compound of sovereign virtue in ginger and its underlying molecular mechanisms responsible have remained unknown. In these reasons, I chose well-studied compounds in ginger and compared their anti-inflammatory effects. Results to compare effects of ginger compounds on monocyte-endothelial adhesion show that gingerenone A has the most suppressive effects among other ginger compounds and curcumin.

Inflammation occurred in early stage of atherosclerosis is stimulated by TNF-α or LPS. LPS induces cascade in NF-κB signaling pathway through toll-like receptors and TNF-α is expressed through

TNFR in inflammatory circumstance. In this study, I demonstrated gingerenone A inhibits binding of monocytes to human endothelial cells, HUVECs, stimulated by TNF-α or LPS dose dependently.

Additionally, VCAM-1 and MCP-1 are dominant molecules expressed in inflammation during progress of atherosclerosis [33]. When monocytes in blood bind to endothelial cells by stimulation of inflammatory action, VCAM-1 is highly expressed in endothelial cells. Also, MCP-1 expression level is increased to recruit more monocytes from blood in inflammatory circumstance. In this research, gingerenone A reduces VCAM-1 and MCP-1 expression in protein as well as mRNA level. Thus, gingerenone A has protective effect in early progress of atherosclerosis.

As it is generally known, NF-κB signaling pathway regulates inflammatory response in atherosclerosis, as well as phosphorylation of IKK, IκBα and translocation of NF-κB affect VCAM-1 and MCP-1

expression [13, 34]. I observed that gingerenone A dose dependently suppressed phosphorylation of IKK and IκBα in human endothelium induced by TNF-α or LPS. However, although IKK is a key factor to regulate inflammation, I could not assure that it is a target to inhibit VCAM-1 and MCP-1 expression. To find out the target protein, further study to check other protein in signaling pathways involved inflammation such as MAPKs and PI3K-Akt signaling pathways should be done.

In summary, these results support that gingerenone A has suppression effects on adherence of monocytes to endothelial cells, on protein and mRNA expression of adhesion molecules and on transcription factor NF-κB action in human endothelial cells stimulated by two different inflammatory factors. I discovered gingerenone A suppresses monocyte-endothelial adhesion appeared in early stage of atherosclerosis by inhibiting VCAM-1 and MCP-1 expression which are

regulated by phosphorylation of IKK. Therefore, gingerenone A might be a potential candidate for the prevention of atherosclerosis.

Ⅴ. References

1. Ross, R., *The pathogenesis of atherosclerosis: a perspective for the 1990s.* Nature, 1993. **362**(6423): p. 801-9.
2. Hansson, G.K., *Inflammation, atherosclerosis, and coronary artery disease.* N Engl J Med, 2005. **352**(16): p. 1685-95.
3. Hansson, G.K. and P. Libby, *The immune response in atherosclerosis: a double-edged sword.* Nat Rev Immunol, 2006. **6**(7): p. 508-19.
4. Cybulsky, M.I., et al., *A major role for VCAM-1, but not ICAM-1, in early atherosclerosis.* J Clin Invest, 2001. **107**(10): p. 1255-62.
5. Iiyama, K., et al., *Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation.* Circ Res, 1999. **85**(2): p. 199-207.
6. Packard, R.R. and P. Libby, *Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction.* Clin Chem, 2008. **54**(1): p. 24-38.
7. Pugin, J., et al., *Lipopolysaccharide activation of human*

endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc Natl Acad Sci U S A, 1993. **90**(7): p. 2744-8.

8. Osborn, L., et al., *Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes.* Cell, 1989. **59**(6): p. 1203-11.
9. McKellar, G.E., et al., *Role for TNF in atherosclerosis? Lessons from autoimmune disease.* Nature Reviews Cardiology, 2009. **6**(6): p. 410-417.
10. Zhang, L., et al., *Expression of tumor necrosis factor receptor-1 in arterial wall cells promotes atherosclerosis.* Arteriosclerosis, thrombosis, and vascular biology, 2007. **27**(5): p. 1087-1094.
11. Li, H. and B. Sun, *Toll-like receptor 4 in atherosclerosis.* Journal of cellular and molecular medicine, 2007. **11**(1): p. 88-95.
12. Bonizzi, G. and M. Karin, *The two NF-κB activation pathways and their role in innate and adaptive immunity.* Trends in immunology, 2004. **25**(6): p. 280-288.
13. Brand, K., et al., *Role of nuclear factor-kappa B in atherogenesis.* Experimental physiology, 1997. **82**(2): p. 297-304.
14. Shu, H.B., et al., *Differential regulation of vascular cell adhesion*

molecule 1 gene expression by specific NF-kappa B subunits in endothelial and epithelial cells. Molecular and cellular biology, 1993. **13**(10): p. 6283-6289.

15. Tak, P.P. and G.S. Firestein, *NF-κB: a key role in inflammatory diseases.* Journal of clinical investigation, 2001. **107**(1): p. 7.

16. Chrubasik, S., M. Pittler, and B. Roufogalis, *Zingiberis rhizoma: a comprehensive review on the ginger effect and efficacy profiles.* Phytomedicine, 2005. **12**(9): p. 684-701.

17. Bode, A.M. and Z. Dong, *The Amazing and Mighty Ginger*, in *Herbal Medicine: Biomolecular and Clinical Aspects*, I.F.F. Benzie and S. Wachtel-Galor, Editors. 2011, CRC Press

18. Gundala, S.R., et al., *Enterohepatic recirculation of bioactive ginger phytochemicals is associated with enhanced tumor growth-inhibitory activity of ginger extract.* Carcinogenesis, 2014. **35**(6): p. 1320-9.

19. Nicoll, R. and M.Y. Henein, *Ginger (Zingiber officinale Roscoe): a hot remedy for cardiovascular disease?* Int J Cardiol, 2009. **131**(3): p. 408-9.

20. Ghayur, M.N., et al., *Cardiovascular effects of ginger aqueous*

extract and its phenolic constituents are mediated through multiple pathways. Vascular pharmacology, 2005. **43**(4): p. 234-241.

21. Fuhrman, B., et al., *Ginger extract consumption reduces plasma cholesterol, inhibits LDL oxidation and attenuates development of atherosclerosis in atherosclerotic, apolipoprotein E-deficient mice.* The Journal of nutrition, 2000. **130**(5): p. 1124-1131.
22. Dugasani, S., et al., *Comparative antioxidant and anti-inflammatory effects of [6]-gingerol,[8]-gingerol,[10]-gingerol and [6]-shogaol.* Journal of ethnopharmacology, 2010. **127**(2): p. 515-520.
23. Venkateswarlu, S., et al., *Synthesis of gingerenone-A and hirsutenone.* Indian Journal of Chemistry, 2001. **40B**: p. 495-497.
24. Nallasamy, P., et al., *Sulforaphane reduces vascular inflammation in mice and prevents TNF-alpha-induced monocyte adhesion to primary endothelial cells through interfering with the NF-kappaB pathway.* J Nutr Biochem, 2014. **25**(8): p. 824-33.
25. Sikora, E., G. Scapagnini, and M. Barbagallo, *Curcumin, inflammation, ageing and age-related diseases.* Immunity & Ageing, 2010. **7**(1): p. 1.

26. Carlos, T., et al., *Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells [published erratum appears in Blood 1990 Dec 1; 76 (11): 2420]*. Blood, 1990. **76**(5): p. 965-970.

27. Maus, U., et al., *Role of endothelial MCP-1 in monocyte adhesion to inflamed human endothelium under physiological flow.* American Journal of Physiology-Heart and Circulatory Physiology, 2002. **283**(6): p. H2584-H2591.

28. Cannon, B., *Biochemistry to behaviour.* Nature, 2013. **493**: p. S2-S3.

29. Lusis, A.J., *Atherosclerosis.* Nature, 2000. **407**(6801): p. 233-41.

30. Stocker, R. and J.F. Keaney, *Role of oxidative modifications in atherosclerosis.* Physiological reviews, 2004. **84**(4): p. 1381-1478.

31. Weber, C., A. Zernecke, and P. Libby, *The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models.* Nat Rev Immunol, 2008. **8**(10): p. 802-15.

32. Alizadeh-Navaei, R., et al., *Investigation of the effect of ginger on the lipid levels. A double blind controlled clinical trial.* Saudi medical journal, 2008. **29**(9): p. 1280-1284.

33. Libby, P., P.M. Ridker, and A. Maseri, *Inflammation and atherosclerosis.* Circulation, 2002. **105**(9): p. 1135-1143.

34. Gareus, R., et al., *Endothelial cell-specific NF-kappaB inhibition protects mice from atherosclerosis.* Cell Metab, 2008. **8**(5): p. 372-83.

Ⅵ. 국문 초록

동맥경화는 발병 초기에 면역세포가 혈관내피 층에 부착할 때 생기는 염증에 의해서 진행된다. 동맥경화의 진전을 예방하기 위해서는 동맥경화의 초기단계를 막는 것이 중요하다. 예로부터 생강은 심혈관계 질환의 치료효능이 있다고 알려져 왔지만 생강 속 특정한 물질들에 한하여 연구가 진행되어 왔고 치료효능의 작용기전 또한 명확하게 밝혀있지 않다. 본 연구자는 다섯 가지의 생강 속 물질을 분석하였고 이를 통해 진저레논 에이가 면역세포와 내피세포의 부착을 가장 잘 예방한다는 것을 알 수 있었다. 또한 연구를 통해 진저레논 에이가 면역세포의 부착에 관여하는 물질인 VCAM-1과 MCP-1의 생성을 효과적으로 억제한다는 것과 진저레논 에이가 동맥경화의 혈관 염증을 조절하는 NF-κB 신호 전달계 중 IKK의 인산화를 억제함을 밝혔다. 종합적으로 이 연구의 결과들을 통해 진저레논 에이가 IKK의 인산화를 억제함으로써 면역세포의 내피세포 부착과 부착과 관련된 물질들의 발현을 줄이는 것을 확인 할 수 있었다.