Genistein Suppression of TNF- α -induced Fractalkine Expression in Endothelial Cells

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Key Words

Genistein • Fractalkine • Endothelial cells

Abstract

Genistein is a polyphenolic nonsteroidal isoflavonoid with estrogen-like activity has been shown to have anticancer, antioxidant, and anti-inflammatory activities. Fractalkine is a unique chemokine that functions as a chemoattractant and an adhesion molecule on endothelial cells activated by proinflammatory cytokines. In this study, we investigated the effects of genistein (5–25 μ M) on fractalkine expression in human umbilical vein endothelial cells (HUVECs) and on its receptor, CX3CR1, in THP-1 cells in response to treatment with tumor necrosis factor (TNF)- α . TNF- α significantly induced fractalkine expression in endothelial cells. Genistein decreased TNF- α -induced fractalkine expression through suppression of Akt and p38 phosphorylation and NF- κ B activities. Genistein also strongly suppressed TNF- α -induced expression of CX3CR1 in monocytes. Genistein suppressed TNF- α -stimulated adhesion of monocytes to HUVECs. Immunohistochemical analysis revealed that genistein

suppressed the *in vivo* lipopolysaccharide (LPS)-induced arterial endothelial fractalkine expression in the heart, kidney, and small intestine. These results suggest that genistein may provide a new pharmacological approach for suppressing fractalkine/CX3CR1-mediated injury under vascular inflammatory conditions.

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Introduction

Fractalkine (CX3CL1), a structurally distinct chemokine, is expressed in activated endothelial cells; its expression is upregulated by tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and lipopolysaccharide (LPS) [1, 2]. Fractalkine exists in a membrane-anchored or shed chemotactic soluble form [3, 4]. A full-length transmembrane version of fractalkine acts as an adhesion molecule and efficiently captures cells under physiological flow conditions [5, 6]. The soluble form of fractalkine acts as a potent chemoattractant for monocytes, T cells, and natural killer cells that express the fractalkine receptor CX3CR1 [7]. Thus, fractalkine and CX3CR1 interaction is important for tethering and rolling, arrest, stable

adhesion, and transendothelial migration of CX3CR1-expressing leukocytes in fractalkine-expressing inflamed endothelium [8]. Fractalkine expression has been shown to be increased in sites of vascular and tissue damage in various diseases such as atherosclerosis [9], cardiac allograft rejection [10], HIV infection [11], rheumatoid arthritis [12], and sepsis [13, 14]. Thus, regulation of fractalkine expression in vascular endothelial cells may be important in preventing and treating inflammation and these diseases.

Genistein is a major isoflavone in soy and red clover, and has various biological actions, including a weak estrogenic effect by binding estrogen receptors and inhibiting protein tyrosine kinases [15]; it also protects against a number of human chronic diseases, including cardiovascular diseases, cancer, and osteoporosis [16-18]. Genistein protects proinflammatory pathways by inhibiting cytokine-induced overexpression of proinflammatory reactions in human brain microvascular endothelial cells [19]. It also suppresses endotoxin-induced inflammatory reactions in the liver and intestines *in vivo* [20]. However, the effects of genistein on fractalkine expression and its signaling pathway in endothelial cells, and on CX3CR1 expression in monocytes and macrophages, have not been examined.

In this study, we investigated whether genistein decreases TNF- α -induced expression of fractalkine in human umbilical vein endothelial cells (HUVECs) and TNF- α -induced CX3CR1 expression in THP-1 cells. We also evaluated the effects of genistein on LPS-induced endothelial fractalkine expression *in vivo*. In addition, we investigated the signal mechanisms that mediate the suppressive effect of genistein on fractalkine expression in endothelial cells.

Materials and Methods

Materials and cell culture

Anti-fractalkine (full length) and anti-CX3CR1 antibodies were purchased from Torrey Pines BioLabs (Houston, TX). Anti-phospho-p65 (Ser536) and anti-p65 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Mitogen-activated protein kinase (MAPK) kinase 1/2 inhibitor PD98059, p38 inhibitor SB203580, and NF- κ B inhibitor pyrrolidine dithiocarbamate were purchased from Calbiochem (San Diego, CA). Genistein, phosphatidylinositol 3-kinase (PI3'-K) inhibitor LY294002, TNF- α , and most other biochemical reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. We prepared HUVECs from human umbilical cords by collagenase digestion as previously

described [16]. The THP-1 cells were from American Type Culture Collection (Manassas, VA).

Western blot analysis

Immunoblotting analysis was performed as described previously [15]. HUVECs and THP-1 cells were treated with genistein for 30 min before treatment with TNF- α (10 ng/mL) for 6 h. The samples (20 μ g of protein per lane) were mixed with sample buffer, boiled for 10 min, separated by sodium dodecyl sulfide-polyacrylamide (10%) gel electrophoresis under denaturing conditions, and electroblotted onto nitrocellulose membranes. The membranes were incubated overnight at 4°C with anti-fractalkine, anti-fractalkine receptor (dilution, 1:2,000) or anti-phospho-p65 (Ser536) (dilution, 1:1,000) antibodies. The membranes were reblotted with anti-actin and anti-p65 antibodies (1:2,000) to verify equal loading of protein in each lane. The signals were visualized and analyzed by densitometric scanning (LAS-3000; Fuji Film, Tokyo, Japan).

Monocyte isolation and adhesion assay

The study protocol and informed consent forms were approved by the Chonbuk National University Hospital Review Board. All patients gave informed consent. Human peripheral blood monocytes were isolated from the fresh blood of healthy volunteers by Ficoll-Paque gradient centrifugation. Monocytes were further purified by negative selection using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocyte-endothelial adhesion was performed according to previously described methods [14].

Flow cytometry

For flow cytometric analysis of fractalkine expression, HUVECs were treated as previously described [21]. HUVECs were treated with genistein for 30 min before treatment with TNF- α (10 ng/mL) for 6 h. The cells were harvested using trypsin and ethylenediamine tetraacetic acid (EDTA) and were washed once with phosphate-buffered saline (PBS) before incubation with an anti-fractalkine antibody (15 min on ice). An FITC-coupled secondary antibody was then added, and cells were further incubated for 15 min on ice. Fractalkine protein expression level was next quantified by fluorescence-activated cell sorting analysis (FACSCalibur; Becton-Dickinson, Franklin Lakes, NJ). A total of 20,000 events were analyzed per sample to calculate the frequency of fractalkine-positive cells and the mean level of fractalkine expression.

Electrophoretic mobility shift assay

Subconfluent cells were pretreated with genistein for 30 min and then stimulated with 10 ng/mL TNF- α for 2 h. Nuclear protein extracts were prepared by using a nuclear extraction kit (Panomics, Redwood City, CA). An electrophoretic mobility shift assay (EMSA) was performed according to a previously described method [22]. We performed an EMSA for NF- κ B using the biotin-labeled NF- κ B binding site oligomer 5'-AGT TGA GGG GAC TTT CCC AGG C-3'. The signals were detected by chemiluminescence imaging according to the manufacturer's protocol (EMSA gel-shift kit; Panomics).

Fig. 1. Genistein suppresses TNF- α -induced fractalkine expression. **A**, Western blot analyses of fractalkine protein in TNF- α -stimulated HUVECs cotreated with genistein (Geni). HUVECs were incubated for 6 h with control buffer (Cont), TNF- α (10 ng/mL), or TNF- α plus the indicated amount of genistein. Western blot was probed with an anti-fractalkine antibody and reprobed with an anti-actin antibody to verify equal loading of protein in each lane. **B**, densitometric analyses are presented as the relative ratio of each protein to actin. The ratio relative to control is arbitrarily presented as 1. Bars represent the mean $\pm$ SD from 3 experiments. *, $p < 0.05$ versus control; #, $p < 0.05$ versus TNF- α only. **C**, analysis of fractalkine expression in HUVECs as detected by flow cytometry. HUVECs incubated with control buffer (Cont), genistein (Geni, 25 μ M), TNF- α (10 ng/mL), or TNF- α (10 ng/mL) plus genistein (25 μ M) for 6 h.

Animal experiments

Inbred male Sprague-Dawley rats (Charles River Korea, Seoul, Korea) were fed a standard laboratory diet and water *ad libitum* and were maintained under a protocol approved by the Institutional Animal Care and Use Committee of the Korea Food Research Institute. The rats (180–200 g) were divided into 3 groups: control [0.1% dimethylsulfoxide (DMSO) ($n = 5$)], LPS (10 mg/kg; $n = 5$), and LPS (10 mg/kg) plus genistein (10 mg/kg; $n = 5$). Genistein was administered by oral gavage once daily for 14 days before LPS administration. Control buffer or LPS were then injected intravenously into a tail vein. Twelve hours after injection of DMSO or LPS, the rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and sacrificed by cervical dislocation. The heart, kidneys, and small intestine were harvested for immunohistochemical analysis.

Immunohistochemistry

Immunohistochemistry of fractalkine was performed as previously described [14]. Isolated heart, kidney, and small intestine tissues were fixed by immersion in 4% paraformaldehyde and embedded in paraffin. Tissues were sectioned at 5 μ m, and 8–12 sections of the heart, kidney, and small intestine were deparaffinized with xylene and rehydrated with ethanol. The sections were incubated overnight at 4°C with an anti-fractalkine antibody (dilution 1:100; R&D Systems, Minneapolis, MN). Signals were visualized with the Cell and

Tissue Staining Kit (R&D Systems). The sections were counterstained with Meyer's hematoxylin and were viewed and photographed with a Zeiss Z1 microscope (Carl Zeiss, Göttingen, Germany). Fractalkine expression was semiquantitated by grading the degree of immunostaining (5 = very strong, 4 = strong, 3 = moderate, 2 = weak, 1 = no staining). Three to five endothelial portions of each section were graded. Two independent, blinded investigators graded the expression by using a charge-coupled device camera. Inter-investigator variation was $<5\%$.

Densitometric analyses and statistics

Data are expressed as mean (standard deviation (SD)). Statistical significance was tested for by use of Student's *t* test or one-way analysis of variance followed by the Student–Newman-Keuls test. Statistical significance was set at $p < 0.05$.

Results

Genistein suppresses fractalkine expression and the number of fractalkine-positive cells in TNF- α -treated HUVECs

We examined the effect of genistein on TNF- α -induced fractalkine expression in HUVECs by western

Fig. 2. Genistein decreases CX3CR1 protein in TNF- α -stimulated THP-1 cells. A, Western blot analyses of CX3CR1 protein in TNF- α -stimulated THP-1 cells cotreated with genistein (Geni). THP-1 cells were incubated for 6 h with control buffer (Cont), TNF- α (10 ng/mL), or TNF- α plus the indicated amount of genistein. Western blot was probed with an anti-CX3CR1 antibody and reprobed with an anti-actin antibody to verify equal loading of protein in each lane. B, densitometric analyses are presented as the relative ratio of each protein to actin. The ratio relative to control is arbitrarily presented as 1. Bars represent mean $\pm$ SD from 3 experiments. *, $p < 0.05$ versus Cont; #, $p < 0.05$ versus TNF- α only. C, analysis of fractalkine expression in HUVECs detected by flow cytometry. HUVECs incubated with control buffer (Cont), genistein (Geni, 25 μ M), TNF- α (10 ng/mL), or TNF- α (10 ng/mL) plus genistein (25 μ M) for 6 h.

blotting and flow cytometry. In the western blot analysis, genistein suppressed TNF- α -induced fractalkine expression in a dose-dependent manner. Genistein at 10 μ M suppressed approximately 40-50% of the TNF- α -induced fractalkine expression, whereas concentrations of 25 and 50 μ M almost completely suppressed TNF- α -induced fractalkine expression (Fig. 1A and B). The number of fractalkine-positive cells was increased approximately 4.5-fold in HUVECs after stimulation with TNF- α . Treatment with genistein and TNF- α decreased the number of positive cells to the control level (Fig. 1C). These data suggest that genistein inhibits TNF- α -induced fractalkine expression in HUVECs.

Genistein reduces CX3CR1 expression in THP-1 cells

We examined the effect of genistein on TNF- α -induced CX3CR1 expression in THP-1 cells. TNF- α increased CX3CR1 expression by approximately 2.0-fold at 6 h. Genistein suppressed TNF- α -induced CX3CR1 expression in a dose-dependent manner. At 10 μ M, genistein suppressed approximately 23% of TNF- α -induced CX3CR1 expression, whereas higher concentrations of genistein (50 μ M) resulted in

suppression to about control levels. Genistein (25 μ M) alone did not significantly affect CX3CR1 levels (Fig. 2). These results indicate that genistein inhibits the TNF- α -induced expression of CX3CR1 in THP-1 cells.

Genistein suppresses TNF- α -induced monocyte adhesion to HUVECs

Ancuta and colleagues [23] reported that the induction of fractalkine expression in endothelial cells induces monocyte adhesion. Therefore, we examined the effect of genistein on monocyte adhesion stimulated by TNF- α in HUVECs. Stimulation of HUVECs with TNF- α (10 ng/mL) for 6 h induced a significant increase (approximately 4-fold) in monocyte adhesion. Genistein inhibited TNF- α -induced monocyte adhesion in a dose-dependent manner: 5 μ M decreased adhesion by approximately 26%, whereas 25 and 50 μ M caused a 50% decrease (Fig. 3). Treatment of TNF- α -stimulated cells with an anti-fractalkine antibody led to a 32% decrease in monocyte adhesion, whereas addition of genistein (25 μ M) blocked monocyte adhesion, compared with cotreatment with genistein (25 μ M) and TNF- α . These findings suggest that genistein suppresses monocyte adhesion to TNF- α -stimulated HUVECs through fractalkine expression.

Fig. 3. Genistein suppresses TNF- α -induced endothelial adhesiveness of monocytes. A, Fluorescence microscopy of endothelial adhesiveness for monocytes. Monocytes were fluorescently labeled with calcein-AM (50 μ M, bright green color). Bars, 50 μ m. B, quantification of monocyte adhesion to HUVECs. Monocytes were added to confluent monolayers of HUVECs, which were treated with TNF- α (10 ng/mL) for 6 h and were also treated with control buffer (Cont), TNF- α plus the indicated amount of genistein (Geni), or anti-fractalkine antibody (20 μ g/mL). Bars represent the mean $\pm$ SD from 5 experiments. *, $p < 0.05$ versus control; #, $p < 0.05$ versus TNF- α ; ##, $p < 0.05$ versus TNF- α plus genistein (25 μ M); ###, $p < 0.05$ versus TNF- α plus anti-fractalkine antibody.

Fig. 4. Inhibitors alter the TNF- α -stimulated expression of fractalkine. A, Western blot analyses of fractalkine protein in TNF- α -stimulated HUVECs cotreated with genistein (Geni) and inhibitors. HUVECs were incubated with TNF- α (10 ng/mL) for 6 h in the presence of control buffer (Cont), genistein (Geni, 25 μ M), pyrrolidine dithiocarbamate (PT, 50 μ M), PD98059 (PD, 50 μ M), SB203580 (SB, 500 μ M), and LY294002 (LY, 10 μ M). Total proteins (20 μ g) isolated from the cells were subjected to Western blot analysis. B, densitometric analyses are presented as the relative ratio of each protein to actin. The ratio relative to control is arbitrarily presented as 1. Bars represent mean $\pm$ SD from 3 experiments. *, $p < 0.05$ versus control; #, $p < 0.05$ versus TNF- α only.

Inhibitors alter the TNF- α -stimulated expression of fractalkine

We previously reported [13, 21] that TNF- α -induced fractalkine expression might be mediated mainly through the activation of NF- κ B and PI3'-K pathways. To examine the second messenger and transcriptional mechanisms leading to induction of fractalkine by

TNF- α , we added various inhibitors to TNF- α -stimulated HUVECs. The NF- κ B inhibitor pyrrolidine dithiocarbamate (50 μ M) suppressed TNF- α -induced expression of fractalkine better than genistein (25 μ M). The p38 inhibitor SB203580 (5 μ M) and PI3'-K inhibitor LY294002 (10 μ M) also suppressed TNF- α -induced fractalkine expression. However, the MEK 1/2 inhibitor

Fig. 5. Genistein suppresses TNF- α -induced Akt and p38 phosphorylation. Western blot analyses of Akt (A) and p38 phosphorylation (C) protein in TNF- α -stimulated HUVECs cotreated with genistein (Geni). HUVECs were incubated with TNF- α (10 ng/mL) for 30 min with control buffer (Cont), TNF- α (10 ng/mL), or TNF- α plus the indicated amount of genistein. Western blot was probed with an anti-phospho-Akt and p38 antibody and reprobed with an anti-Akt and p38 antibody to verify equal loading of protein in each lane. Densitometric analyses are presented as the relative ratio of each protein to Akt (B) and p38 (D). The ratio relative to control is arbitrarily presented as 1. Bars represent mean $\pm$ SD from 3 experiments. *, $p < 0.05$ versus control; #, $p < 0.05$ versus TNF- α only.

Fig. 6. Genistein suppresses TNF- α -induced NF- κ B activities. A, Western blot analyses of phospho-p65 subunit of NF- κ B in protein extracts of HUVECs. HUVECs were pretreated with genistein (Geni, 25 μ M) for 30 min and treated TNF- α (10 ng/mL) at the indicated dose for 4 h. B, densitometric analyses are presented as the relative ratio of each protein to the p65 subunit of NF- κ B. The ratio relative to control is arbitrarily presented as 1. Bars represent the mean $\pm$ SD from 3 experiments. *, $p < 0.05$ versus control; #, $p < 0.05$ versus TNF- α only. C, HUVECs were pretreated with genistein (Geni, 25 μ M) for 30 min and then treated with TNF- α (10 ng/mL) at the indicated time. D, densitometric analyses are presented as the relative ratio of each protein to the p65 subunit of NF- κ B. The ratio relative to control is arbitrarily presented as 1. Bars represent the mean $\pm$ SD from 3 experiments. *, $p < 0.05$ versus control; #, $p < 0.05$ versus TNF- α only. E, EMSA of NF- κ B in nuclear extracts of HUVECs. HUVECs were incubated with control buffer (Cont), genistein (Geni, 25 μ M), or TNF- α plus genistein for 2 h. F, densitometric analyses are presented as the relative ratio of each protein to the p65 subunit of NF- κ B. The ratio relative to control is arbitrarily presented as 1. Bars represent the mean $\pm$ SD from 3 experiments. *, $p < 0.05$ versus cont; #, $p < 0.05$ versus TNF- α only.

PD98059 did not cause any change (Fig. 4). These results suggest that TNF- α -stimulated fractalkine expression might be mediated mainly by activation of NF- κ B through the PI3'-K and p38 pathways.

Genistein suppresses TNF- α -induced Akt and p38 phosphorylation

As shown in Figure 4, TNF- α stimulated the expression of fractalkine through the Akt and p38 pathways in HUVECs. Therefore, we examined the

effect of genistein on the TNF- α -stimulated phosphorylation of Akt and p38 in HUVECs. TNF- α treatment significantly increased Akt and p38 phosphorylation, which was suppressed by genistein in a dose-dependent manner. Genistein at 25 μ M completely blocked TNF- α -stimulated Akt and p38 phosphorylation (Fig. 5). These results suggest that genistein suppresses fractalkine expression by inhibiting Akt and p38 phosphorylation in HUVECs.

Fig. 7. Genistein suppresses LPS-induced fractalkine expression *in vivo*. A, immunohistochemical analyses of arterial endothelial fractalkine expression in the rat heart, kidney, and small intestinal wall. Rats were given control buffer (CB), LPS (10 mg/kg), or LPS plus genistein (Geni, 50 mg/kg per day). Scale bar = 50 μ m. B, semiquantitative analysis of fractalkine expression in the heart, kidney, and small intestine. For each section, 3-5 endothelial portions of the tissue were graded on a scale from 1 (no staining) to 5 (very strong). Bars represent the mean $\pm$ SD from 3 independent experiments. *, $p < 0.05$ versus CB; #, $p < 0.05$ versus LPS only.

Genistein suppresses TNF- α -induced NF- κ B activity

We examined the effect of genistein on NF- κ B activity using a phosphospecific anti-p65 antibody that only detects the p65 subunit of NF- κ B when it is phosphorylated at Ser536. TNF- α induced the phosphorylation of the p65 subunit (approximately 2.8-fold). Genistein inhibited TNF- α -induced phosphorylation of the p65 subunit in a dose-dependent manner; this phosphorylation was nearly completely inhibited by cotreatment of genistein (25 μ M) and TNF- α (Fig. 6A and B). Also, 25 μ M genistein inhibited TNF- α -induced phosphorylation of the p65 subunit in a time-dependent manner, with maximal suppression at 4 h after genistein treatment (Fig. 6C and D); genistein alone had no effect. To identify the NF- κ B binding complexes induced by TNF- α , nuclear extracts of HUVECs were analyzed by EMSA. As shown in Figure 6E and F, NF- κ B (p65/p50) binding was increased in nuclear extracts of TNF- α -stimulated HUVECs, whereas it was suppressed by TNF- α and genistein cotreatment. These data suggest that genistein suppresses fractalkine expression

by inhibiting NF- κ B activity in HUVECs.

Genistein suppresses LPS-induced fractalkine expression in endothelial cells of the heart, kidney, and small intestine

We examined the effect of LPS on fractalkine expression in the rat heart, kidney, and small intestine using immunohistochemistry. Similar to the result obtained in our previous report [14], intravenous injection of LPS (10 mg/kg) markedly increased fractalkine expression at 12 h in arterial endothelial cells of the heart. In the kidneys, LPS treatment increased fractalkine expression in arterial endothelial cells but not in glomerular endothelial or tubular epithelial cells. In the small intestine, LPS increased fractalkine expression in arteriolar endothelial cells and villous endothelium, but not in lymphatic endothelial cells or epithelial cells. Pretreatment with genistein (10 mg/kg per day) dramatically suppressed LPS-induced fractalkine expression in arterial endothelial cells in the heart, kidney, and small intestine (Fig. 7). These findings suggest that genistein suppresses LPS-induced fractalkine expression in endothelial cells of the heart, kidney, and small intestine.

Discussion

In this study, we demonstrated that genistein suppressed TNF- α -induced fractalkine expression through the suppression of Akt and p38 phosphorylation and NF- κ B activities in endothelial cells; inhibited TNF- α -induced fractalkine receptor expression in monocytes; suppressed TNF- α -stimulated monocyte adhesion to HUVECs; and decreased LPS-induced arterial endothelial fractalkine expression in the heart, kidney, and intestinal wall. These results suggest that genistein may have potential to be used as a novel pharmacological agent for suppressing fractalkine/CX3CR1-mediated injury under inflammatory conditions.

Vascular endothelial cells can be important pathogenic factors in inflammatory disorders such as sepsis. Sepsis is a clinical syndrome that represents a systemic response to infection and is characterized by a systemic widespread inflammatory response that triggers organ damage and ultimately organ failure [24]. Sepsis is caused mainly by an exaggerated systemic response to endotoxemia caused by gram-negative bacteria and their characteristic cell wall component, LPS. In mice, challenge with high doses of LPS results in a syndrome that resembles septic shock in humans [25]. We previously reported that fractalkine increases the induction of TNF- α in HUVECs [21]. In this study, we showed that genistein strongly inhibits TNF- α -induced fractalkine expression in endothelial cells (Fig. 1) and LPS-induced fractalkine expression in endothelial cells of the heart, kidney, and intestinal wall (Fig. 7). The important biological roles of fractalkine in endothelial cell inflammation and injury may involve mediating the adherence of leukocytes in CX3CR1-expressing cells. Recently, Oh and colleagues [26] reported that CX3CR1 inhibition protects against ischemic acute renal failure by reducing macrophage infiltration in mice. In this study, we have shown that genistein suppressed CX3CR1 expression in monocytes after stimulation with TNF- α (Fig. 2). We also demonstrated that genistein decreased TNF- α -induced adhesion of human monocytes to HUVECs (Fig. 3). These results suggest that genistein could be useful for preventing fractalkine-mediated vascular inflammatory injury. Because fractalkine and CX3CR1 play critical roles in inflammation, therapeutic interventions that target fractalkine in endothelial cells and CX3CR1 in monocytes or macrophages may potentially control inflammatory diseases.

Activation of NF- κ B may play a central role in inflammatory cytokine-induced fractalkine expression [27]. Our previous data showed that TNF- α -induced

expression of fractalkine occurs mainly through the activation of the NF- κ B and PI3-K pathways [28]. Recently, we reported that genistein protects against cisplatin-induced renal injury via the NF- κ B signaling pathway [22]. Further, Lu and colleagues reported that genistein reduces the production of pro-inflammatory and adhesion molecules induced by hemolysate in brain microvascular endothelial cells [29]. Si et al. recently reported that genistein inhibits TNF- α -induced apoptosis through p38 β MAPK in endothelial cells [30]. In this study, we demonstrated that genistein suppressed TNF- α -induced Akt and p38 phosphorylation (Fig. 5) and NF- κ B binding activity in endothelial cells (Fig. 6). Therefore, we suggest that genistein plays a regulatory role in fractalkine expression through the suppression of Akt and p38 phosphorylation and NF- κ B binding.

The excessive production of proinflammatory cytokines is thought to contribute significantly to the lethality of sepsis. Proinflammatory TNF- α and IL-1 β act as initiators in the cascade of endogenous mediators that direct the inflammatory reaction, eventually leading to severe shock [27, 31]. Among the inflammatory mediators, fractalkine is upregulated by TNF- α and LPS [14]. As you pointed out, the peaking of the plasma level of TNF- α at 2 h ($2,000 \pm 450$ pg/mL) and the gradual decline thereafter observed in this study were similar to the findings of our previous study [14]. In addition, Zhao et al [32] reported that genistein decreased the serum level of TNF- α at 2 h after LPS treatment. However, in this study, we focused on the effect of genistein on LPS-induced fractalkine expression, and therefore we treated rats with LPS for 12 h; this is the time required for TNF- α -induced fractalkine expression. Thus, at 12 h, the serum level of TNF- α reduced to the baseline levels.

Most studies show that blood concentrations of genistein, over the range of typical human and rodent consumption, are <10 μ M, and genistein is quickly cleared from the circulation [30, 33-34]. However, in this study, to examine the effect of genistein on LPS-induced fractalkine expression in HUVECs, the cells were treated with different genistein concentrations (5-50 μ M). Furthermore, to determine the specific effects, a 25- μ M dose was used. We observed that genistein suppressed TNF- α -induced fractalkine expression, Akt and p38 phosphorylation, and NF- κ B activities in a dose-dependent manner. In addition, genistein dose-dependently suppressed the TNF- α -induced expression of CX3CR1 in monocytes and adhesion of monocytes to HUVECs. Modest changes in fractalkine expression, as observed following exposure to relatively low concentrations of

genistein (5-10 μ M), may contribute to a significant reduction in vascular inflammation. Moreover, exposure to genistein in the range of 5-10 μ M was found to effectively suppress fractalkine expression. Thus, the genistein concentrations that produced biological effects in this study (5-25 μ M) partially overlap with the concentrations attainable in plasma and tissues in humans following dietary supplementation. Moreover, genistein circulates as glucuronidated and sulfated metabolites in plasma [35]. The formation of glucuronide and sulfate conjugates is likely to reduce the inflammatory effect of genistein since important functional groups of the genistein molecule are masked. Therefore, further studies are required to determine the physiologically achievable concentrations of genistein and their metabolites and their effects on endothelial cell function.

In conclusion, our results indicate that genistein exerts an anti-inflammatory effect via a novel mechanism - the downregulation of fractalkine and CX3CR1 expression. Thus, genistein is a potential agent for adjunctive therapy in inflammatory disease.

Acknowledgements

This study was supported by research grants Korea Research Foundation for the Biofoods Research Program, Ministry of Education, Science & Technology, Inter-Institutional Collaboration Research Program under the Korea Research Council for Industrial Science and Technology (KOCT), and by the Korea Food Research Institute, Korea.

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