Cyanidin and Cyanidin-3-O-β-D-glucoside Suppress the Inflammatory Responses of Obese Adipose Tissue by Inhibiting the Release of Chemokines MCP-1 and MRP-2

Mi-Ran Choe¹, Ji-Hye Kang¹, Hoon Yoo², Suck-Young Choe¹†, Chae Ha Yang³, Mi-Ok Kim⁴, and Rina Yu¹

¹Department of Food Science and Nutrition, University of Ulsan, Ulsan 680-749, Korea
²Department of Pharmacology and Dental Therapeutics, College of Dentistry, Chosun University, Gwangju, Korea
³Department of Physiology, College of Oriental Medicine, Daegu Haany University, Daegu, Korea
⁴Department of Health Diet, Daegu Health College, Daegu, Korea

Abstract

Obesity-induced inflammation plays a crucial role in obesity-related pathologies such as type II diabetes and atherosclerosis. Adipose tissue macrophages and the cell-derived proinflammatory chemokines are key components in augmenting inflammatory responses in obesity. Anthocyanins such as cyanidin and cyanidin-3-O-β-D-glucoside (C3G) are known to elicit anti-inflammatory activities by suppressing the production of proinflammatory mediators such as tumor necrosis factor alpha and nitric oxide in LPS-stimulated macrophages. In the present study, we investigated whether cyanidin and C3G have the potential to suppress the inflammatory responses of adipose cells. Cyanidin and C3G not only suppressed the migration of RAW 264.7 macrophages induced by mesenteric adipose tissue-conditioned medium, but also inhibited the activation of the cells to produce inflammatory chemokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-related protein-2 (MRP-2) in a dose-dependent manner. Cyanidin and C3G also inhibited the release of MCP-1 and MRP-2 from adipocytes and/or macrophages. These findings suggest that cyanidin and C3G may suppress the inflammatory responses of adipose tissue in obesity.

Key words: anthocyanins, obesity-induced inflammation, macrophage, chemokine, adipose tissue

INTRODUCTION

Obesity-induced inflammation is a major risk factor of obesity-related pathologies such as type II diabetes and atherosclerosis. Adipose tissue macrophages and the cell-derived proinflammatory chemokines are key components in augmenting inflammatory responses in obesity. Anthocyanins such as cyanidin and cyanidin-3-O-β-D-glucoside (C3G) are known to elicit anti-inflammatory activities by suppressing the production of proinflammatory mediators such as tumor necrosis factor alpha and nitric oxide in LPS-stimulated macrophages. Therefore, modulating the chemokine production in adipose tissue may contribute to suppression of the inflammatory responses of adipose tissue macrophages.

Anthocyanins, naturally occurring phytochemicals, are a group of phenolic compounds related to the coloring of plants, flowers and fruits and thus widely consumed in daily diet. Anthocyanins elicit antioxidative and anti-inflammatory properties. For example, delphinidin, cyanidin, and cyanidin 3-O-β-D-glucoside (C3G) suppress the production of proinflammatory mediators such as nitric oxide and prostaglandin E2 through nuclear factor-kappa B inactivation in lipopolysaccharide-stimulated macrophages (8,9). In addition, cyanidin and C3G have been shown to improve hyperlipidemia and hyperglycemia in mice fed a high-fat diet (10,11). These findings suggest that the anthocyanins may improve the inflammatory micro-

†Corresponding author. E-mail: sychoe@ulsan.ac.kr
Phone: +82-52-259-2373, Fax: +82-52-259-1699
environment in obese adipose tissue, and thereby attenuate the development of obesity-related pathologies. However, little is currently known about whether the anthocyanins suppress obesity-induced inflammatory responses. In this study, we investigated whether C3G and cyanidin suppress the inflammatory responses of adipose tissue by inhibiting macrophage migration and inflammatory chemokine productions. Our data demonstrated that cyanidin and C3G have the potential to suppress adipose tissue inflammatory responses in obesity.

**MATERIAL AND METHOD**

**Chemicals**
Cyanidin and cyanidin-3-O-β-D-glucose were purchased from Extrasynthese (Genay, France).

**Cell culture**
The murine macrophage cell line RAW264.7 was obtained from the Korean Cell Line Bank (KCLB40071, Seoul, Korea). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine serum, 10 μg/L penicillin-streptomycin (Gibco BRL, NY, USA), and 2 mg/L gentamicin (Gibco BRL, NY, USA) at 37°C and 5% CO₂. 3T3-L1 pre-adipocytes (ATCC, USA) were cultured in a basal medium consisting of DMEM supplemented with 200 μM ascorbic acid, 10% fetal bovine serum, 10 μg/L penicillin-streptomycin at 37°C in a 5% CO₂ atmosphere. Two days after reaching confluence, the cells were incubated in a differentiation medium containing an inducing mixture (0.25 μM dexamethasone, 10 μg/mL insulin, and 0.5 mM 1-methyl-3-isobutylxanthine) in the basal medium. After 40 hr~42 hr, the cell culture medium was changed to a maturation medium containing 5 μg/mL insulin with the basal medium, and the maturation medium was changed to a fresh medium every 2 days for 6 days.

**Coculture of adipocytes and macrophages**
Differentiated 3T3-L1 adipocytes were cultured in 6 wells, and RAW 264.7 cells (7.5×10⁵ cell/well) were plated onto 3T3-L1 adipocyte for 2 hr. The cells were cultured with or without various anthocyanin and incubated for 24 hr.

**Preparation of mesenteric adipose tissue-conditioned medium**
Male C57BL/6 mice (8 wks, Hyochang Ltd., Daegu, Korea) were fed a 45% high-fat diet for 3 months, and mesenteric adipose tissue (n = 6) was isolated. All subsequent procedures were performed under a laminar flow hood. Adipose tissue was minced into fragments less than 10 mg in weight and cultured as previously described (7). In brief, 50 mg of minced adipose tissue fragments was seeded in 2 mL of serum medium in each well of a 6-well plate. The plate containing the tissue fragments was placed in a humidified incubator at 37°C and 5% CO₂. The medium was supplemented with glutamine, 25 mM HEPES, 50 μg/mL gentamicin and 0.5 μg/mL amphotericin B. The tissue culture was incubated for 72 hr. Aliquots of the culture medium were stored at -80°C until the use.

**Migration assay**
The migration of macrophages was measured by a multiwell microchemotaxis chamber (NeuroProbe Inc., Gaithersburg MD, USA) (7). Briefly, the above prepared RAW264.7 cells were suspended in M199 medium at 1×10⁶ cells/mL and 27 μL was placed in the upper wells of a 96-well chamber that was separated from the lower wells by an 8-μm polycarbonate filter containing adipose tissue-conditioned medium with or without anthocyanin. After incubation for 6 hr at 37°C, nonmigrated cells were removed by scraping them out and cells that migrated across the filter were fixed and stained with Diff-Quik (Merck Corp, Darmstadt, Germany). Stained cells were counted under light microscopy in three randomly chosen high-power fields (HPF; 400×). Results are expressed as mean (±SEM) from triplicate samples. Adipose tissue-conditioned medium-induced cell migration served as a positive control and was considered to show 100% migration for each experiment.

**Measurement of MCP-1 and MRP-2 by ELISA**
The levels of MCP-1 and MRP-2 in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (7,13). ELISA was conducted utilizing the OptEIA™ Mouse MCP-1 set (Pharmingen) or MRP-2 (R&D systems, MN, USA). The concentration of MCP-1 in culture supernatants was determined by ELISA. ELISA was conducted utilizing the OptEIA™ Mouse MCP-1 set (Pharmingen). Samples were thawed, diluted properly in assay diluents, and thoroughly mixed. The samples were pipetted into appropriate wells of MCP-1 antibody-coated ELISA plates. The plates were sealed and incubated for 2 hr at room temperature. After rinsing thoroughly, each well was incubated for 1 hr with 100 μL of a biotinylated mouse MCP-1 monoclonal antibody and an avidin-horseradish peroxidase conjugate. Following another rinse, TMB substrate solution was placed in each well in the dark for 30 min. The absorbance at 450 nm was measured using a microplate reader. The concentrations of MCP-1 were quantified from a standard curve using the SOFTmax curve-fitting pro-
Fig. 1. Inhibitory effects of cyanidin and C3G on macrophage migration induced by mesenteric adipose tissue-conditioned medium. RAW 264.7 cells were placed in the upper wells of a 96-well culture chamber that were separated from the lower wells containing adipose tissue-conditioned media with or without cyanidin and/or C3G and incubated for 6 hr at 37°C. The cells that migrated across the filter were fixed and stained with Diff-Quick, and counted under a light microscope in three randomly chosen high-power fields. Mesenteric adipose tissue-conditioned medium induced cell migration served as positive control and was designated as 100% migration for each determination. Values are means±SEM from three independent experiments. *p<0.05, **p<0.01, significantly different from control.

Statistical analysis
Results are expressed as mean±SEM. Statistical analysis was performed using ANOVA and Dunnett’s multiple comparison test. Differences were considered to be significant at p<0.05.

RESULTS
Cyanidin and C3G inhibit macrophage migration induced by mesenteric adipose tissue-conditioned medium
To test whether cyanidin and C3G inhibit macrophages migration, we treated macrophages with mesenteric adipose tissue-conditioned medium with or without cyanidin and C3G. Mesenteric adipose tissue-conditioned medium containing MCP-1 (7) was used to determine the enhanced inflammatory response of adipose tissue macrophage. As shown in Fig. 1, the mesenteric adipose tissue conditioned medium significantly enhanced macrophage migration and both cyanidin and C3G significantly suppressed the migration of macrophages in a dose-dependent manner (Fig. 1).

Cyanidin and C3G inhibits the production of inflammatory chemokines from macrophages stimulated by mesenteric adipose tissue-conditioned medium
To determine whether the cyanidin and C3G inhibit macrophage activation to produce inflammatory chemokines, we treated macrophages with mesenteric adipose tissue-conditioned medium with or without cyanidin and C3G, and the production levels of the chemokines, MCP-1 and MRP-2, were measured by ELISA. As shown in Fig. 2, the mesenteric adipose tissue-conditioned medium significantly induced RAW264.7 macrophages to release MCP-1 and MRP-2, and cyanidin and C3G decreased MCP-1 and MRP-2 releases from the macrophages stimulated by mesenteric adipose tissue-conditioned medium. The inhibitory effect by cyanidin and C3G was stronger for MCP-1 production than for MRP-2 production.

Cyanidin and C3G inhibits the production of inflammatory chemokines from adipocytes or adipocytes cocultured with macrophages
To determine whether cyanidin and C3G suppress MCP-1 and MRP-2 productions from adipocytes, we treated 3T3-L1 adipocytes with or without cyanidin and C3G. The anthocyanins cyanidin and C3G significantly inhibited MCP-1 and MRP-2 releases from 3T3-L1 adipocytes (Fig. 3). We also treated the adipocytes cocultured with macrophages with or without cyanidin and C3G, and measured the production of MCP-1 and MRP-2. Both compounds markedly decreased MCP-1
Fig. 2. Inhibitory effect of cyanidin and C3G on production levels of MCP-1 and MRP-2 from macrophages stimulated by mesenteric adipose tissue-conditioned medium. RAW 264.7 cells were stimulated with the mesenteric adipose tissue-conditioned medium and incubated for 24 hr with or without cyanidin and/or C3G. The amount of MCP-1 or MRP-2 released in culture medium was measured by ELISA. Values are means±SEM. Representative results of four independent experiments are shown. *p<0.05, **p<0.01, ***p<0.001, significantly different from control.

Fig. 3. Inhibitory effect of cyanidin and C3G on production of MCP-1 and MRP-2 from adipocytes. 3T3-L1 adipocytes were treated with or without cyanidin and/or C3G and incubated for 24 hr. The amounts of MCP-1 and MRP-2 released from 3T3-L1 adipocytes were measured by ELISA. Figure shows the means±SEM of triplicate determinations from one experiment. *p<0.05, **p<0.01, ***p<0.001, significantly different from control.
Fig. 4. Inhibitory effect of cyanidin and C3G on production of MCP-1 and MRP-2 from adipocytes cocultured with macrophages. 3T3-L1 adipocytes were cocultured with RAW 264.7 macrophages (7.5×10^3 cell/well) with or without cyanidin and C3G, and incubated for 24 hr. The amounts of MCP-1 and MRP-2 release from adipocytes or macrophages cocultured with adipocytes were measured by ELISA. Figure shows the means±SEM of duplicate determinations from one experiment. *p<0.05, **p<0.01, ***p<0.001, significantly different from control.

DISCUSSION

In this study, we demonstrated that anthocyanins such as cyanidin and C3G have the potential to suppress adipose tissue inflammatory responses in obesity by modulating the migration of macrophages and the production of chemokines from adipocytes and/or macrophages.

Obesity-induced inflammatory responses are characterized by increased inflammatory mediator production from adipose tissue and macrophage accumulation into adipose tissue (1,7). MCP-1 and MRP-2, CC chemokines, play a pivotal role in various inflammatory processes by regulating leukocyte trafficking to sites of tissue damage. In several recent reports, macrophage as well as adipose tissue and/or adipocyte were found to be a source of various chemokines (e.g., MCP-1, MIP-1a, MRP-2, IL-8) (6,7,14) and the adipose tissue-derived chemokines augmented macrophage infiltration and activation in adipose tissues in obesity (7). We found that cyanidin and C3G directly inhibited macrophage migration induced by adipose tissue-conditioned medium. We also observed that cyanidin and C3G inhibited MCP-1 and MRP-2 productions from macrophages stimulated with mesenteric adipose tissue-conditioned medium. These findings indicate that the anthocyanins can suppress the inflammatory responses of adipose tissue by not only suppressing macrophage migration into adipose tissue, but also inhibiting macrophage activation to produce the inflammatory chemokines such as MCP-1 and MRP-2. Moreover, we found that cyanidin and C3G suppressed MCP-1 and MRP-2 productions from adipocytes, and adipocytes cocultured with macrophages, suggesting that cyanidin and C3G effectively suppress the paracrine interactions between adipocytes and macrophages occurring through chemokines, and thereby attenuate the inflammatory responses of adipose tissues in obesity. Cyanidin, an aglycon of C3G (Fig. 5), is more hydrophilic than C3G. The different effects between cyanidin and C3G suggest that the hydroxyl group on the R-position in the molecular structure of cyanidin may be important for its greater anti-inflammatory activities in adipose tissue. A previous study demonstrated that cyanidin and C3G inhibit IL-6 and inflammatory adipo-
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Fig. 5. Chemical structure of anthocyanins.

R= OH; cyanidin
R= - o-β-D-glucose; cyanidin 3-glucoside (C3G)

In conclusion, anthocyanins such as cyanidin and C3G suppressed the migration of macrophage to adipose tissue and the activation the cells to produce inflammatory chemokine MCP-1 and MRP-2. Cyanidin and C3G also inhibited the production of MCP-1 and MRP-2 from adipocytes and adipocytes cocultured with macrophages, indicating that the cyanidin and C3G suppress the inflammatory responses of adipose tissue in obesity. The anthocyanins may be useful for ameliorating obesity-induced inflammatory responses.

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