RESEARCH ARTICLE

Cyanidin-3-Galactoside Attenuates Glycated LDL-Induced Monocyte Adhesion, Inflammatory Mediators and Endoplasmic Reticulum Stress in Vascular Endothelial Cells

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List of abbreviation:

C3Ga: cyanidin-3-galactoside
C3G: cyanidin-3-glucoside
D3G: delphinidin-3-glucoside
ER: endoplasmic reticulum
EC: endothelial cells
FCS: fetal calf serum
gLDL: glycated low-density lipoprotein
HBSS: Hank's buffered salt solution
HUVEC: human umbilical vein endothelial cells
ICAM-1: intracellular cell adhesion molecule-1
LRP: low density lipoprotein receptor-like protein
MCP-1: monocyte chemotatic protein-1
MyD88: myeloid differentiation primary response gene 88
PAI-1: plasminogen activator inhibitor-1
ThT: thioflavin T
TLR4: toll-like receptor 4
TNFα: tumour necrosis factor-α
uPA: urokinase plasminogen activator
uPAR: uPA receptor

Abstract

Background: Anthocyanins are potent phyto-antioxidants with cardioprotective effects. Cyanidin-3-galactoside (C3Ga) is an anthocyanin abundant in dark-skin fruits, but its effects on vascular inflammation remains unclear.

Purpose: The present study aims to determine the effects of C3Ga on monocyte adhesion to endothelial cells (EC) and relevant mechanism.

Methods: Monocyte adhesion was assessed by incubation of THP-1 human monocytes with human umbilical vein EC exposed to glycated low density lipoprotein (gLDL). Inflammatory, fibrinolytic mediators and endoplasmic reticulum (ER) stress in EC were analyzed using Western blotting or immunoassays.

Results: C3Ga dose-dependently inhibited gLDL-induced monocyte adhesion to EC. C3Ga reduced gLDL-induced increases in the intensity of plasminogen activator inhibitor-1, urokinase plasminogen activator (uPA), uPA receptor, LDL receptor-like protein , intracellular cell adhesion molecule-1, monocyte chemotactic protein-1, tumor necrosis factor-α, toll-like receptor-4, and myeloid differentiation primary response gene-88 and attenuated the intensity of thioflavin T fluorescence, a biomarker of ER stress, in EC. The inhibitory effects of C3Ga on monocyte adhesion, inflammatory mediators and ER stress were substantially weaker than cyanidin-3-glucoside, but comparable to that of delphinidin-3-glucoside.

Conclusion: The results suggest that C3Ga or photo-products enriched with this anthocyanin may help to suppress vascular inflammation and ER stress under diabetic condition.

Key words: Cyanidin-3-galactoside; monocyte adhesion; endothelial cells; inflammatory and fibrinolytic mediators; endoplasmic reticulum stress.

Introduction

The prevalence of diabetes has rapidly increased in most of countries. In 2015, one of 11 adults in the world has been diagnosed as diabetes [1]. The most common cause of death in diabetic patients is cardiovascular complications. Diabetes increases the risk of cardiovascular diseases by 2-4-times [2]. Mechanism for the acceleration of the development of cardiovascular disease in diabetic patients remains unclear. Hyperglycemia and dyslipoproteinemia are two biochemical markers of diabetes. The level of glycated low-density lipoprotein (gLDL) is frequently elevated in diabetic patients [3]. Our previous studies demonstrated that gLDL, a biochemical marker of diabetic condition, can induce oxidative stress, mitochondrial dysfunction, or endoplasmic reticulum (ER) stress, and reduces the expression of endothelial nitric oxide synthase, and enhances monocyte adhesion and the expression of inflammatory mediators in cultured vascular endothelial cells (EC) [4-8]. Increased oxidative stress is associated with endothelial dysfunction, inflammation and atherogenesis. Pharmacological or nutritional intervention which is capable to prevent chronic inflammation and endothelial dysfunction is highly demanded.

Saskatoon berry powder inhibited monocyte adhesion in leptin receptor-deficient diabetic and obese mice [9]. Cyanidin-3-glucoside (C3G), represents 25% of anthocyanins in Saskatoon berry power, suppressed gLDL induced oxidative stress, apoptosis (5), and ER stress in cultured EC [6]. Cyanidin-3-galactoside (C3Ga) is a type of common anthocyanin and a structural homologue of C3G. C3Ga reduced the expression of ER stress mediators in EC, but its effects were weaker than that of C3G [6]. C3Ga represents 74% of anthocyanins in Saskatoon berry powder [9]. The effects of C3Ga on monocyte adhesion, inflammatory mediators or the intensity of ER stress in EC remain unclear.

The present study investigated the effects of C3Ga on monocyte adhesion, inflammatory mediators and thioflavin T (ThT) fluorescent intensity in cultured human vascular EC compared to two other common anthocyanins, C3G and delphinidin-3-glucoside (D3G).

Methods

Cell culture

Human umbilical vein EC (HUVEC) and THP-1 human monocyte seed cells were obtained from American Type Culture Collections (Manassas, VA). HUVEC were cultured in F12K medium (Gibco Invitrogen Canada, Burlington, ON) containing 10% of fetal calf serum (FCS), heparin and EC growth supplements (Sigma-Aldrich Canada, Oakville, ON) as previously described [3]. THP-1 monocytes were grown in RPMI-1640 medium containing 10% of FCS and 0.05 mM 2-mercaptoethanol as instructed by the supplier.

Isolation and modification of lipoproteins

LDL (density 1.019-1.063) was isolated from healthy donors' plasma using sequential density floatation ultracentrifugation. LDL was glycated using 50 mM glucose and 50 mM sodium cyanoborohydride at 37°C for 2 weeks [10]. Free small molecular chemicals in the LDL preparations were removed through overnight dialysis. The glycation of LDL was assessed using trinitrobenzenesulfonic acid assay as previously described [8]. Lipoproteins were stored in sealed tubes under a layer of nitrogen at 4°C in dark to prevent auto-oxidation. Lipoproteins were excluded from experiments if the levels of endotoxin in lipoproteins were above 0.05 ng/mL measured using E-Toxate kit (Sigma) [10].

Monocyte adhesion assay

HUVEC were grown to confluence in 6-well culture dishes. Human THP-1 monocytes (5 x 10^4) were added to each well containing confluent EC. THP-1 monocytes were incubated with EC at 22ºC for 30 min on a rotating mixer. Non-adhered monocytes were removed by 2 washes with Hank's buffered salt solution (HBSS). Adhered monocytes on EC surface were fixed with 2% of glutaraldehyde (Sigma) in HBSS [8]. Monocytes adhered on EC were

observed under light microscopy with 10X magnification. Five distinguished fields in each dish were counted and the averages of adhered monocytes in the fields were used in data analyses as described [8].

Western blotting analysis

Targeted proteins in total cellular proteins of EC were assessed using Western blotting analysis as previously described [11]. Monoclonal or polyclonal antibodies against human of plasminogen activator inhibitor-1(PAI-1), urokinase plasminogen activator (uPA), uPA receptor (uPAR), LDL receptor-like protein (LRP), monocyte chemotatic protein-1 (MCP-1), intracellular cell adhesion molecule-1 (ICAM-1), tumour necrosis factor-α (TNFα)**,** toll-like receptor 4 **(**TLR4), myeloid differentiation primary response gene 88 (MyD88) or β-actin were obtained from Santa Cruz (Santa Cruz, CA) or Abcam (Cambridge, MA). Corresponding secondary antibodies conjugated with horse-radish peroxidase (Santa Cruz) and enhanced chemilluminence reagents (GE Healthcare, Buckinghamshire, UK) were used to visualize specific antigens on nitrocellulose membranes. The abundance of antigens was assessed using Chemi-Doc system and Quantity One software (BioRad, Hercules, CA), and normalized with the abundance of β-actin in corresponding samples.

ThT fluorimetric assay

ThT assay was used to assess misfolded or unfolded proteins in ER stress. After experimental incubation, cellular media were replaced by 1.5 mM ThT in 50 mM glycine NaOH buffer (pH 8.5) for 5 min at 37°C. ThT fluorescence intensity was assessed using a FLUOStar OPTIMA plate reader (BMG Lab Technologies, GmbH, Ortenberg, Germany) with 450/485-nm excitation/emission filters set as previously described [6].

Statistics

One-way ANOVA and Newman-Keuls post-hoc test was performed for comparisons of data among multiple groups. The level of significance was defined as $p<0.05$.

Results

Effects of C3Ga on gLDL-induced monocyte adhesion to EC

The dose-dependence of C3Ga on gLDL-induced monocyte adhesion to HUVEC was examined in the present study. Glycated LDL at 100 μg/mL (a physiological concentration in human) enhanced the adhesion of monocytes to EC as previously optimized (8). The inhibition of C3Ga on gLDL-induced monocyte adhesion was detected at 10 μM compared to no addition ($p<0.05$). The maximal inhibition of C3Ga on gLDL-induced monocyte adhesion was detected at 100 μM ($p<0.01$). The inhibitory effect of C3Ga on gLDL-induced monocyte adhesion was evidently weaker than C3G. The maximal inhibitory effect of C3G on gLDL-induced monocyte adhesion was detected at 30 μM (Fig.1B), which suggests that C3G is a stronger inhibitor for gLDL-induced monocyte adhesion compared to C3Ga. C3Ga or its structural homologue, D3G, another common anthocyanin in dark skin berries, at 100 μM or C3G at 30 μM without an exposure to gLDL did not significantly alter monocyte adhesion to EC. C3Ga or D3G at 100 μM and C3G at 30 μM significantly reduced gLDL-induced monocyte adhesion to EC compared to gLDL ($p<0.01$) (Fig.1A and 1C).

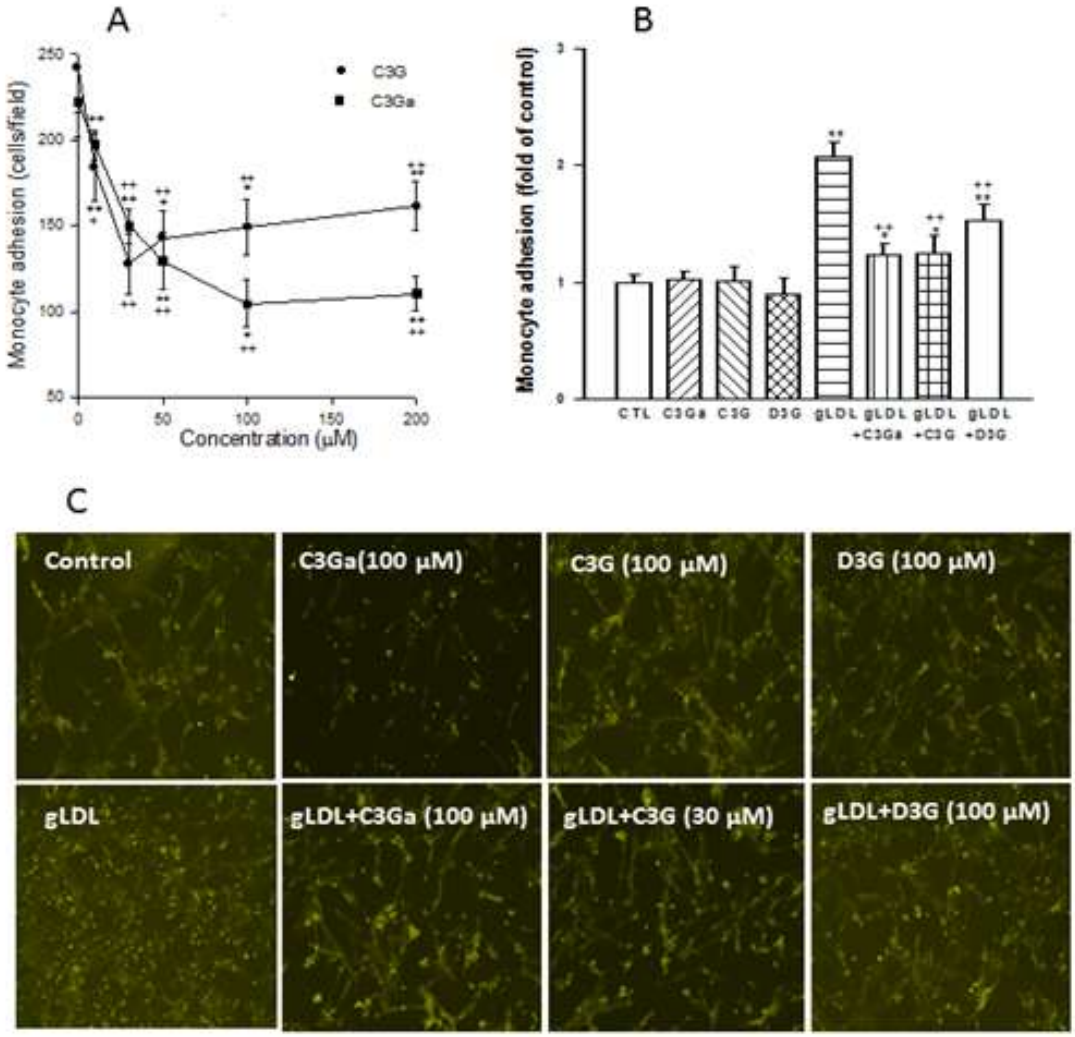

Fig.1 Effects of cyanidin-3-galactoside (C3Ga), cyanidin-3-glucoside (C3G) and delphinidin-3-glucoside (D3G) on monocyte adhesion to glycated LDL (gLDL)-treated endothelial cells (EC). A: Human umbilical vein EC (HUVEC) was treated with 30-200 μM C3Ga or C3G in the presence of 100 μg/mL of gLDL for 6 h at 37ºC under 5% CO_2. B: EC were treated with 100 μM C3Ga or D3G, or 30 μM C3G for 6 h with and without the addition of 100 μg/mL of gLDL. After the removal of anthocyanin containing medium, EC were incubated with THP-1 monocytes for 30 min in room temperature. Attached monocytes were removed by washing. Monocytes adhered to EC were counted under microscopy. Values represents monocytes/field (mean ± SD, n = 3 experiments). *, **: $p<0.05$ or 0.01 versus control (CTL); +, ++: $p<0.05$ or 0.01 versus gLDL; x, xx: $p<0.05$ or 0.01 versus C3G at same concentration.

Effects of C3Ga on the abundances of PAI-1 and uPA in gLDL-treated EC

Previous study demonstrated that PAI-1 and uPA play important roles in gLDL-induced monocyte adhesion to EC (8). C3Ga alone did not significantly affect the levels of PAI-1 or uPA in EC, but it significantly suppressed the increase of PAI-1 or uPA induced by gLDL at the concentrations ≥30 μM ($p<0.05$ or 0.01), and the maximal inhibition on the increases of PAI-1 or uPA was detected in HUVEC treated with 100 μM C3Ga. (Fig.2A). Treatment of EC with 30 μM C3G or 100 μM D3G significantly inhibited gLDL-induced increases in the abundances of PAI-1 or uPA in EC ($p<0.05$ or 0.01, Fig.2B and 2C).

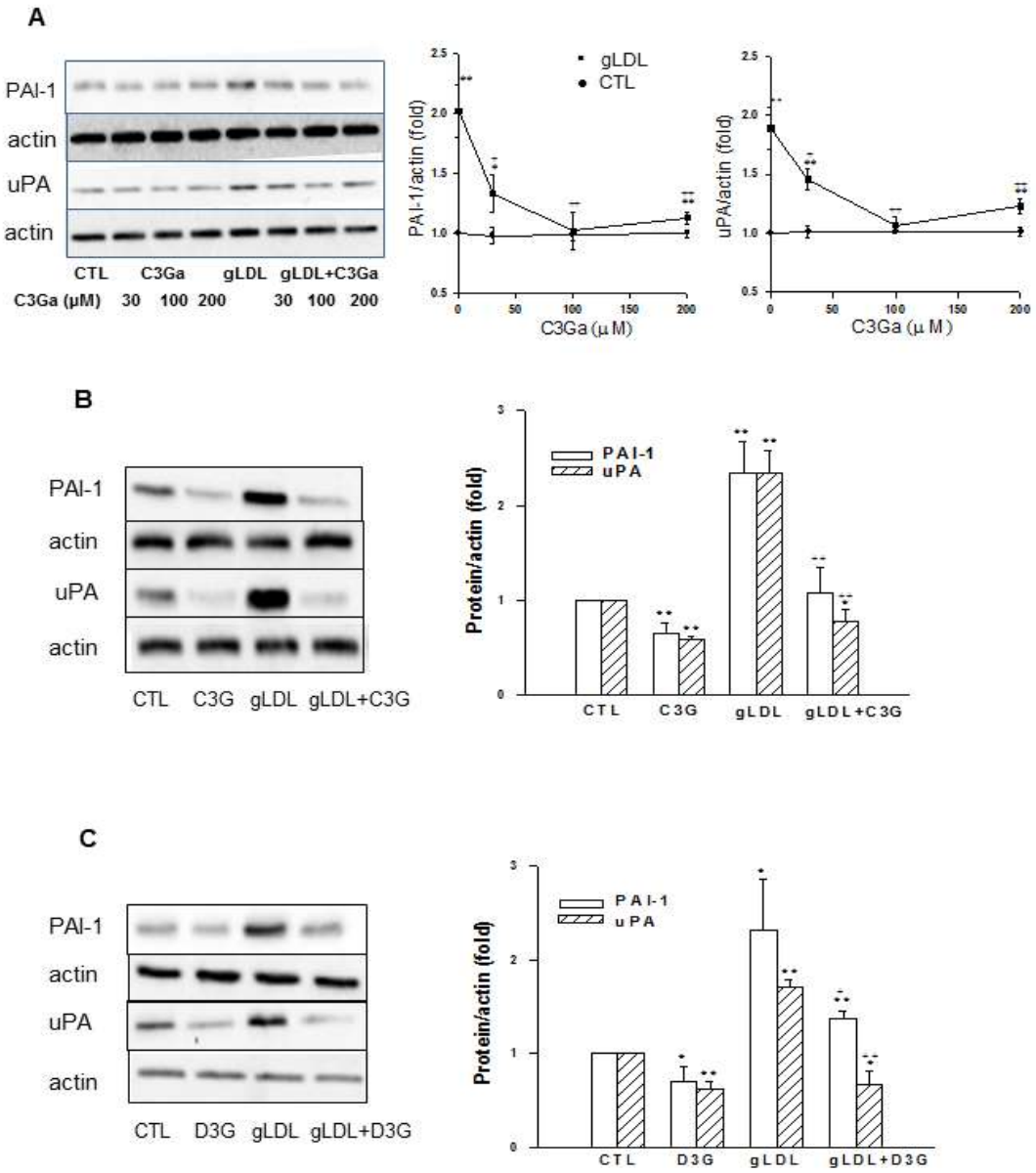

Fig.2 Effects of C3Ga, C3G and D3G on the abundances of plasminogen activator inhibitor-1 (PAI-1) and urokinase plasminogen activator (uPA) in EC. A: HUVEC were treated with vehicle 30-200 μM C3Ga with presence of 100 μg/mL of gLDL for 24 h for PAI-1 or 6 h for uPA. B: HUVEC were treated with vehicle (CTL or control), 30 μM C3G, 100 μg/mL of gLDL or co-treatment of gLDL with C3G for 24 h for PAI-1 or 6 h for uPA assessment. C: HUVEC were treated with vehicle (CTL or control), 100 μM D3G, 100 μg/mL of gLDL or co-treatment of gLDL with D3G for 24 h for PAI-1 or 6 h for uPA assessment. PAI-1 and uPA protein in EC were analyzed using Western blotting. The densities of protein bands were quantified using density scanning. Values were expressed in fold of control (mean ± SD, n = 3 experiments). *,**: $p<0.05$ or 0.01 versus CTL; +,++: $p<0.05$ or 0.01 versus gLDL.

Effects of C3Ga on gLDL-induced uPAR and LRP in EC

The PAI-1/uPA complex may be incorporated into EC via uPAR or LRP, two membrane receptors on EC surface [8]. The effects of C3Ga on gLDL-induced increases in the abundances of uPAR and LRP in HUVEC were examined in comparison to that of C3G and D3G. Glycated LDL increased the expression of uPAR and LRP in EC ($p<0.05$ or 0.01). C3Ga and D3G at 100 μM, or C3G at 30 μM, significantly reduced gLDL-induced increases in uPAR and LRP or at basal conditions in EC ($p<0.05$ or 0.01, Fig. 3A-3C).

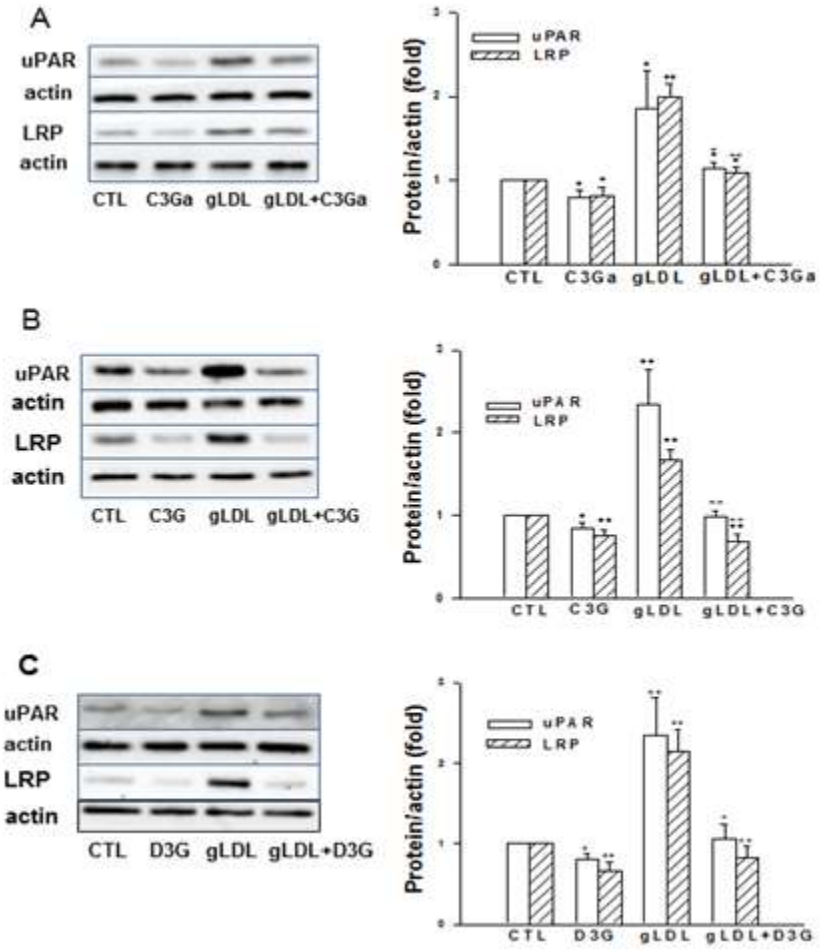

Fig.3 Effects of C3Ga, C3G and D3G on the abundances of uPA receptor (uPAR) and LDL receptor-like protein (LRP) in EC. HUVEC were treated with vehicle (CTL or control), 100 μM C3Ga, 30 μM C3G or 100 μM D3G with and without the addition of 100 μg/mL of gLDL for 6 h. uPAR and LRP protein in EC were analyzed using Western blotting. The density of protein bands were quantified using density scanning. A: C3Ga; B: C3G; C: D3G. Values were expressed in fold of control (mean ± SD, n = 3 experiments). *,**: $p<0.05$ or 0.01 versus CTL; +,++: $p<0.05$ or 0.01 versus gLDL.

Effects of C3Ga on gLDL-induced increases in ICAM-1 and MCP-1 in EC

ICAM-1 and MCP-1 are implicated in gLDL-induced chemotaxis and the rolling over of monocytes on endothelial surface [8]. The present study demonstrated that the treatment of EC with 30 μM C3G, 100 μM C3Ga or D3G significantly inhibited baseline or gLDL-induced increases in the abundances of ICAM-1 and MCP-1 in EC ($p<0.05$ or 0.01, Fig. 4A-4C).

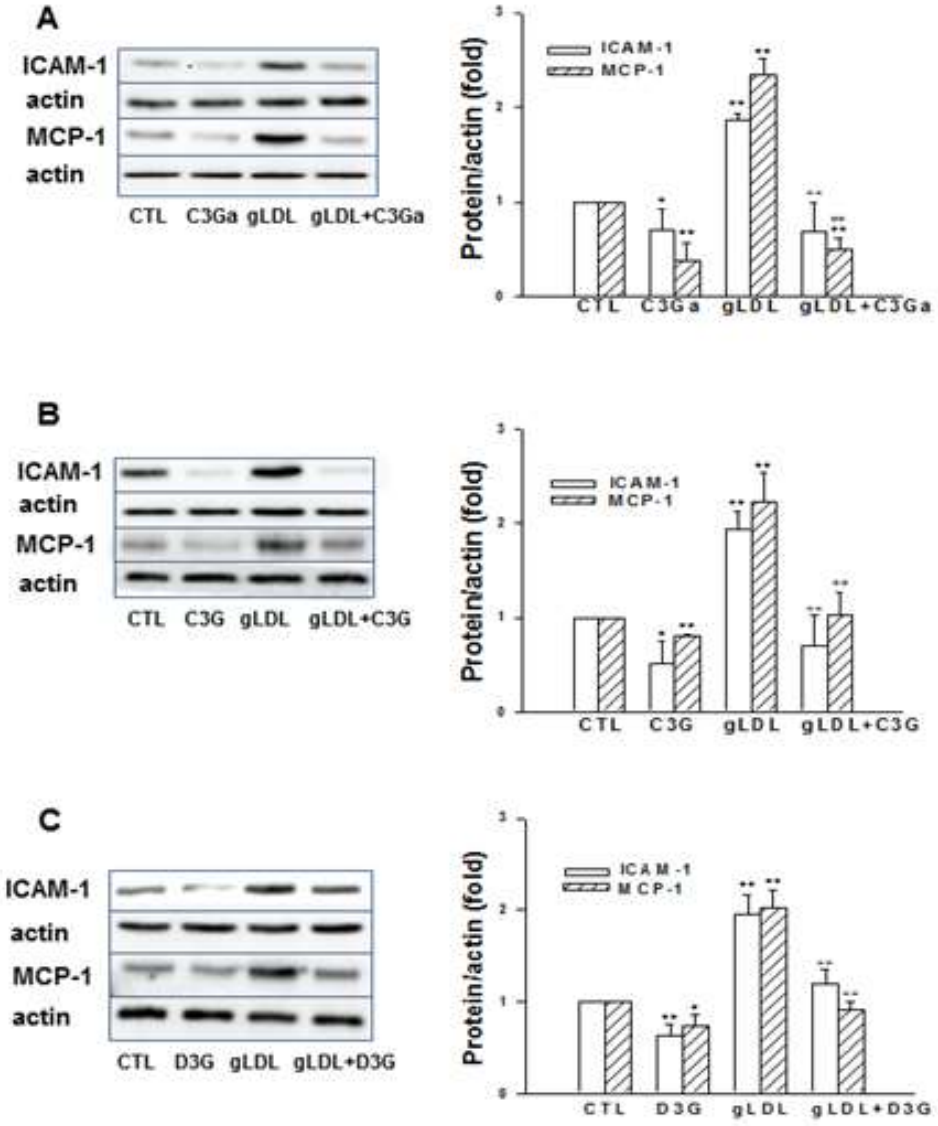

Fig.4 Effects of C3Ga, C3G and D3G on the abundances of intracellular cell adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) in EC. HUVEC were treated with vehicle (CTL or control), 100 μM C3Ga, 30 μM C3G or 100 μM D3G, with and without the addition of 100 μg/mL of gLDL for 6 h. ICAM-1 and MCP-1 protein in EC were analyzed using Western blotting. The densities of protein bands were quantified using density scanning. A: C3Ga; B: C3G; C: D3G. Values were expressed in fold of control (mean ± SD, n = 3 experiments). *,**: $p<0.05$ or 0.01 versus CTL; +,++: $p<0.05$ or 0.01 versus gLDL.

Effects of C3Ga on gLDL-induced TNFα, TLR4 and MyD88 in EC

Our previous studies demonstrated that transfection of TNFα or TLR4 siRNA to HUVEC inhibited gLDL-induced monocyte adhesion to EC [18]. The effects of C3Ga on gLDL-induced increases in the abundances of TNFα, TLR4 and MyD88 in HUVEC were examined and compared to those of C3G and D3G. C3Ga and D3G (100 μM) or C3G (30 μM) significantly reduced basal and gLDL-induced increases in TNFα, TLR4 and MyD88 in EC ($p<0.05$ or 0.01, Fig. 5A-5C).

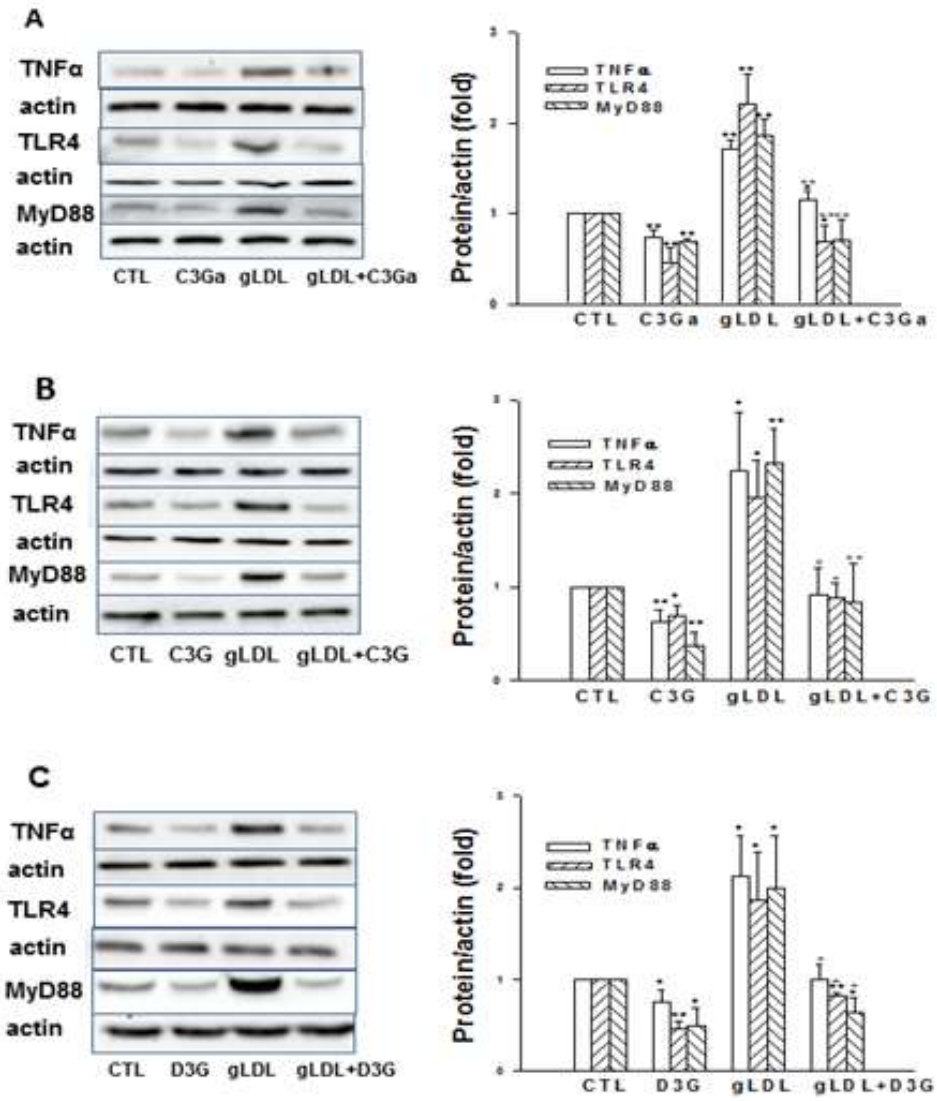

Fig.5 Effects of C3Ga, C3G and D3G on the abundances of tumor necrosis protein-α (TNFα), toll-like receptor-4 (TLR4) and myeloid differentiation primary response gene 88 (MyD88) in EC. HUVEC were treated with vehicle (CTL or control), 100 μM C3Ga, 30 μM C3G or 100 μM D3G with and without addition of 100 μg/mL of gLDL for 6 h. TNFα, TLR4 and MyD88 proteins in EC were analyzed using Western blotting. A: C3Ga; B: C3G; C: D3G. Values were expressed in fold of control (mean ± SD, n = 3 experiments). *,**: $p<0.05$ or 0.01 versus CTL; +,++: $p<0.05$ or 0.01 versus gLDL.

Effects of C3Ga on gLDL-induced increase in ThT intensity in EC

Previous studies demonstrated that ER stress is implicated in leukocyte adhesion to airway epithelial cells [12]. The effects of C3Ga, C3G and D3G on gLDL-induced increases in ThT intensity, a marker for unfolded protein response in ER stress [13], in HUVEC were examined in the present study. The results demonstrated that gLDL significantly increase THT fluorescence in EC compared to control culture ($p<0.01$). Treatment with 100 μM C3Ga, D3G or 30 μM C3G significantly suppressed gLDL-induced increase in the intensity of ThT fluorescence in EC ($p<0.01$), but the anthocyanins did not significantly alter the fluorescent density of ThT in EC at basal condition (Fig. 6).

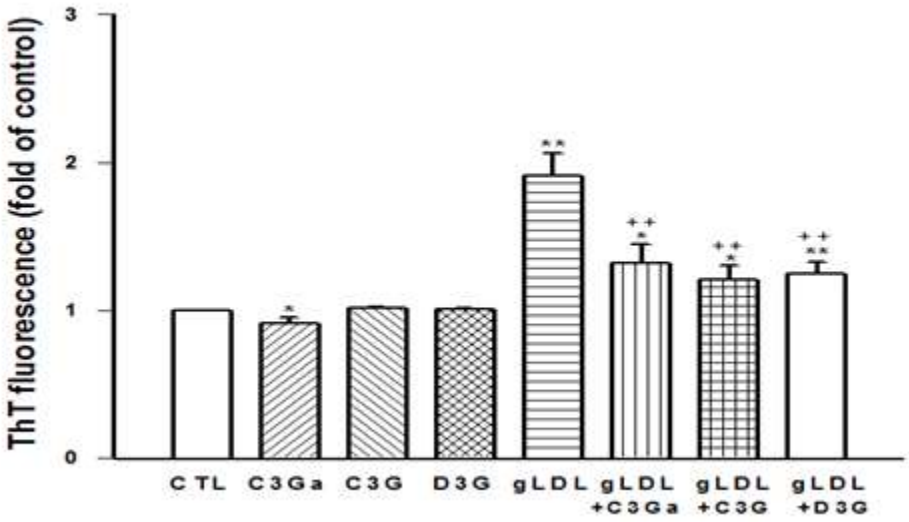

Fig.6 Effects of C3Ga, C3G and D3G on the intensity of thioflavin-T (ThT) in EC. HUVEC were treated with vehicle (CTL or control), 100 μM C3Ga, 30 μM C3G or 100 μM D3G with and without addition of 100 μg/mL of gLDL for 6 h. Unfolded protein response in EC were assessed using ThT assay. Values were expressed in the fold of control (mean ± SD, n = 3 experiments). *,**: $p<0.05$ or 0.01 versus CTL; ++: $p<0.01$ versus gLDL.

Discussion

related to monocyte adhesion, including PAI-1, uPA, uPAR, LRP, ICAM-1, MCP-1, TNFα, TLR4 and MyD88, in EC; 3) C3Ga attenuates the increase of ThT intensity in EC induced by gLDL; 4) the inhibitory effects of C3Ga on monocyte adhesion, fibrinolytic or inflammatory mediators and ThT intensity are weaker than C3G, but comparable to that of D3G.

C3Ga is the dominant anthocyanin in Saskatoon berry [6], chokeberry juice [14], cocoa bean [15] or Starkrimson apple [16]. Krga et al. demonstrated that the exposure of HUVEC to multiple anthocyanins including C3Ga and their metabolites (protocatechuic, vanillic, ferulic and hippuric acid) reduced TNFα-induced monocyte adhesion to EC, but the relationship between the anti-inflammatory of C3G or its metabolites with other anthocyanins and other inflammatory mediators in EC were not assessed in that study [17]. The present study demonstrates following major novel findings: 1) C3Ga dose-dependently inhibits gLDL-induced monocyte adhesion to EC; 2) C3Ga reduces gLDL-induced augments in the levels of a group of fibrinolytic or inflammatory mediators (MCP-1 and ICAM-1) and fibrinolytic regulators or membrane-associated proteins (PAI-1, uPA, uPAR and LRP) in EC [8, 18]. In addition, the present study demonstrated that C3Ga, as well as C3G and D3G inhibited gLDL-induced increases in TNFα, TLR4 and MyD88 in EC. EC-derived TNFα may stimulate the expression of PAI-1, uPA, MCP-1 and ICAM-1 [19-22]. TLR4 mediates the transmembrane activation associated with TNFα. MyD88 is associated with TLR4 activation. The results of the present study suggest that TNFα/TLR4/MyD88 pathway is involved in gLDL-induced inflammatory responses, and multiple anthocyanins in Saskatoon berry may suppress monocyte adhesion to EC induced by gLDL mediated by this pathway [23].

Various species or cultivars of berries contain different amounts of anthocyanins, which may relate to their anti-inflammatory capacities. The present study demonstrated that the anti-inflammatory activities of C3Ga were around 30% of that of C3G, and were similar to equimolar concentration of D3G, in gLDL-treated EC. However, C3Ga represents 74.5% of anthocyanin in Saskatoon berry, which was more than 3-time of that of C3G [6]. The results suggest that total capacity of C3Ga in Saskatoon berry contribute to the inhibitory effect on monocyte adhesion to EC at least in a similar extent compared to that of C3G.

ER stress is characterized by the accumulation of unfold or misfolded proteins in cells, which interrupts normal cellular functions. ER stress often associated with oxidative stress and has been implicated in many pathological processes including inflammation, apoptosis, mitochondrial dysfunction, cancer, endothelial dysfunction, diabetic vascular complications and atherosclerosis [24-27]. Polyphenols have been considered as a class of potential candidates for preventing ER stress under diabetic condition [28]. Our previous studies demonstrated that C3Ga inhibited gLDL-induced increases in the abundances of the markers of unfold protein response, GRP78/94, XBP-1 and CHOP, in EC [6]. The results of the present study demonstrated that C3Ga substantially reduced gLDL-induced increase in ThT intensity, a marker of unfolded protein response in ER stress. The finding of the present study supports our previous study on the effects of C3Ga on the unfolded protein responses [6] The efficacy of C3Ga on ER stress is weaker than C3G, but was comparable to that of D3G. The results suggest that C3Ga is one of anthocyanins capable to inhibit ER stress under diabetic condition.

Diabetes is difficult to be cured and requires long-time, often life-time, of medical care. Glucose lowering therapies have been widely applied in diabetes patients, but the treatments are often associated with adverse effects [29]. Natural foods and nutraceuticals with anti-inflammatory activities are safe and effective alternatives or supplemental therapy for diabetic patients to prevent and manage diabetic cardiovascular complications. The results of the present study suggest that C3Ga and plant products enriched with C3Ga, C3G or D3G are potentially to be used as nutraceuticals or functional foods for preventing chronic inflammation and diabetic cardiovascular complications.

In conclusion, the present studies for the first time displayed that treatment with C3Ga inhibited gLDL-induced monocyte adhesion, the increases of the abundance of relevant inflammatory mediators, related membrane proteins and ER stress marker in EC. The inhibitory effect of C3Ga on vascular inflammation may result from its suppression on the activation of TNFα/TLR4/MyD88 pathway, the expression of fibrinolytic regulators-related membrane proteins and ER stress in EC. Fruits or vegetables abundant in C3Ga may be beneficial for preventing vascular inflammation in individuals with risk for diabetes or diabetic patients.

Contributions of authors: R.Z. contributed to the experimental data for monocyte adhesion, Western blotting analysis and cell culture, and was involved in the draft of manuscript. X.X. was responsible to the ER stress analysis. G.X.S. contributed to project design and manuscript writing.

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