Supplementation of Cyanidin-3-O-β-Glucoside Promotes Endothelial Repair and Prevents Enhanced Atherogenesis in Diabetic Apolipoprotein E–Deficient Mice1–3

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Abstract
Atherosclerosis is accelerated in diabetes mellitus mainly due to the reduced availability and function of endothelial progenitor cells (EPCs). The purpose of this study was to determine the protective effects of the anthocyanin cyanidin-3-O-β-glucoside (C3G) on EPC function and endothelial repair in diabetic apolipoprotein E–deficient (apoE−/−) mice. Diabetes mellitus was induced in 8-wk-old male apoE−/− mice with streptozotocin. Diabetic apoE−/− mice were fed the AIN-93 diet or an AIN-93 diet supplemented with C3G (0.2% wt/wt) for 6 wk. Sham-injected apoE−/− mice fed the AIN-93 diet served as nondiabetic controls. The endothelium-dependent relaxation response to acetylcholine in the aortas of C3G-fed mice was greater by 51% compared with diabetic mice fed the AIN-93 diet (P < 0.05) and was similar to that in nondiabetic apoE−/− mice. The capacity of in vitro adhesion to fibronectin, migration, and tube formation was significantly impaired in diabetic EPCs (decreased by 83, 61.9, and 74.5%, respectively, compared with nondiabetic controls; all P < 0.05) and was similar to that in nondiabetic apoE−/− mice. The purpose of this study was to determine the protective effects of the anthocyanin cyanidin-3-O-β-glucoside (C3G) on EPC function and endothelial repair in diabetic apolipoprotein E–deficient (apoE−/−) mice. Diabetes mellitus was induced in 8-wk-old male apoE−/− mice with streptozotocin. Diabetic apoE−/− mice were fed the AIN-93 diet or an AIN-93 diet supplemented with C3G (0.2% wt/wt) for 6 wk. Sham-injected apoE−/− mice fed the AIN-93 diet served as nondiabetic controls. The endothelium-dependent relaxation response to acetylcholine in the aortas of C3G-fed mice was greater by 51% compared with diabetic mice fed the AIN-93 diet (P < 0.05) and was similar to that in nondiabetic apoE−/− mice. The capacity of in vitro adhesion to fibronectin, migration, and tube formation was significantly impaired in diabetic EPCs (decreased by 83, 61.9, and 74.5%, respectively, compared with nondiabetic controls; all P < 0.05). At the molecular level, the phosphorylation levels of AMP-activated protein kinase (AMPK) Thr172 and endothelial nitric oxide synthase (eNOS) Ser1177 were increased by 3.9-, 2-, and 1.8-fold compared with diabetic EPCs, respectively; all P < 0.05). The capacity of in vitro adhesion to fibronectin, migration, and tube formation was significantly impaired in diabetic EPCs (decreased by 83, 61.9, and 74.5%, respectively, compared with nondiabetic controls; all P < 0.01), which was significantly rescued in response to C3G (increased by 3.9-, 2-, and 1.8-fold compared with diabetic EPCs, respectively; all P < 0.05). At the molecular level, the phosphorylation levels of AMP-activated protein kinase (AMPK) Thr172 and endothelial nitric oxide synthase (eNOS) Ser1177 were higher in EPCs derived from the C3G-treated diabetic mice compared with those in nondiabetic mice. Furthermore, compared with nondiabetic controls, diabetic apoE−/− mice had a 3.5-fold increase in the aortic lesion area, which was lowered by 45% in C3G-fed diabetic mice. This study extends our current knowledge that C3G improves the impairment of EPC function, enhances endothelial repair, and thus limits accelerated atherogenesis caused by diabetes. Our findings emphasize the potential utility of anthocyanin in the prevention and treatment of diabetic vascular complications. J. Nutr. 143: 1248–1253, 2013.

Introduction
Endothelial dysfunction plays a key role in the pathogenesis of diabetic vascular disease (1,2). The mechanisms of the endothelial dysfunction in diabetes are mainly due to the imbalance of endothelial damage and endothelial repair (3,4). Endothelial progenitor cells (EPCs)6 are bone marrow–derived cells with the potential to differentiate into mature endothelial cells and take part in endothelial repair and maintenance under physiologic conditions (5,6). In response to vascular damage, EPCs are mobilized from bone marrow into the circulation, differentiate into functional endothelial cells, and subsequently migrate to the area of injury to facilitate re-endothelialization (6). Diabetic vascular complications may be in part due to the inability of EPCs to exert their reparative function (7). Diabetic EPCs display functional impairments, including decreased migration/homing process, reduced proliferative capacity, and incorporation into tubular structures (8–10). In addition, the number of circulating EPCs is also significantly decreased in diabetic patients and has been considered to be a biological marker of atherosclerosis in diabetes (11,12). Strategies to increase circulating EPC numbers and to improve EPC function are essential to ensure the therapeutic viability of diabetic-induced vascular disorders.

Increasing evidence shows that foods rich in anthocyanins, such as blueberries, purple grapes, and corn, may help prevent cardiovascular disease, particularly with respect to their antioxidant...
and antiinflammatory effects (13–15). Many studies have fo-
cused on cyanidin-3-O-β-glucoside (C3G), the best-known and 
most investigated anthocyanin (16). We recently showed that 
C3G supplementation significantly improved endothelial dys-
fuction and attenuated atherosclerotic lesion progression in
hypercholesterolemic apolipoprotein E-deficient (apoE<sup>-/-</sup>) mice 
(17), the underlying cause of which may associated with the
induction of oxysterol efflux from endothelial cells by anthocyan-
in (18). Despite these promising findings, the protective role
of anthocyanin on diabetes-accelerated atherosclerosis, which
is characterized by the disruption of endothelial repair, remains
poorly demonstrated. The purpose of this study was to investigate
whether anthocyanin C3G is sufficient to prevent diabetes-
induced impairment in EPC functions and vascular repair in
diabetic apoE<sup>-/-</sup> mice.

Materials and Methods

Materials. Anthocyanin C3G was provided by Polyphenol AS.
Streptozotocin and other reagents were purchased from Sigma-Aldrich
unless otherwise indicated.

Animal protocol and diet. Male homozygous apoE<sup>-/-</sup> mice (C57BL/6
 genetic background) were obtained at 6 wk of age from Jackson
Laboratories. The mice were fed an AIN-93 purified diet (19) and given
access to water and food throughout the study. After 2 wk of
acclimatization, the mice were rendered diabetic by 5 consecutive daily i.p.
injections of streptozotocin (Sigma-Aldrich) at a dose of 55 mg
kg<sup>-1</sup>·d<sup>-1</sup> (20). Control mice received vehicle (citrate buffer, pH 4.4) alone.
Diabetes was verified on the basis of a blood glucose concentration
>16.7 mmolL. Diabetic mice were randomly assigned to receive the AIN-93
diet or an AIN-93 diet supplemented with 0.2% (wt:wt) C3G. Non-
diabetic apoE<sup>-/-</sup> mice fed the AIN-93 diet served as controls. The dietary
intervention lasted for 6 wk. The dose of C3G was the same as that
described earlier (17). Body weight and food intake were recorded
weekly. At the end of experiment, mice were killed by using CO<sub>2</sub>,
and the mean value of 5 random fields was de-

Measurement of blood glucose and cholesterol. Blood samples
were collected from the lateral saphenous vein at the end of the study.
Blood glucose and serum lipid concentrations were measured as previously
described (17).

Vascular reactivity. The endothelium-dependent relaxation response to
acetylcholine and the endothelium-independent relaxation response to
sodium nitroprusside were examined as previously described (17).

Quantification of atherosclerotic lesion area. Atherosclerosis was
determined by cross-sectional assessment of the aortic sinus.
The base of each heart was cryosectioned and stained as previously described (17).

Immunohistochemical analysis. Aortas were fixed in 4% buffered
formalin, cut to 5-µm thick sections, and stained for the endothelial cells
marker CD31. Briefly, paraffin-embedded sections were incubated with
anti-CD31 antibody (1:50; Abcam), followed by biotinylated goat anti-
rabbit antibody, and then colored with diaminobenzidine. Sections
were counterstained with hematoxylin.

Isolation of EPCs. EPCs were isolated from blood and bone mar-
row of both nondiabetic and diabetic apoE<sup>-/-</sup> mice. Mononuclear
cells were isolated from blood with Histopaque 1083 (Sigma-Aldrich)
by density gradient centrifugation, and RBCs were lysed with Red
Blood Cell Lysis Buffer (Beyotime). Cells were washed 3 times in Stain
Buffer (BD Pharamingen) and then used for testing by flow cytometry.
Bone marrow-derived EPCs were isolated and cultured according to pre-
viously described methods (21). Briefly, mononuclear cells were obtained
by flushing the femurs and tibias of the mice. Immediately after isolation,
cells were plated onto fibronectin (Sigma)-coated 6-well plates at a
density of 5 × 10<sup>4</sup> cells/well and cultured in Endothelial Cell Growth
Medium 2 (EGM-2; Lonza) supplemented with 20% FBS (Hyclone) in 37°C
5% CO<sub>2</sub>. After 4 d in culture, nonadherent cells were washed away, and fresh
medium was added. EPCs after 7 d in culture were used for all experiments.

Flow cytometry analysis. The number of circulating EPCs was
determined by quantification of the number of Sca-1/Flk-1 double-
positive cells by fluorescence-activated cell sorter (22). In brief, the cells
were incubated with rat anti-mouse Sca-1-FTTC (fluorescein isothio-
 cyanate) antibody (BD) and rat anti-flk-1-phycocerythrin antibody
(BD). The FTTC or PE-conjugated isotype antibody was used as a
control. Data were analyzed by using FACSDiVa software (BD).

EPC adhesion assay. For adhesion assay, 1.0 × 10<sup>6</sup> EPCs were plated
in 96-well plates coated with 1.0 μg/mL fibronectin. After 1-h incubation
in EGM-2 plus 10% FBS, nonadherent cells were washed away, and
adherent cells were fixed with 4% paraformaldehyde. Nuclei were
stained with Hoechst 33258 and were counted at 5 random fields in each
well, at a magnification ×100 for each sample (23).

EPC migration assay. EPC migration was measured by using Transwell
chambers (Corning) with 8-µm-pore-size filters. EPCs were plated at a
density of 5 × 10<sup>5</sup> cells per well in the upper chamber with 200 μL
of EBM-2 (Lonza), whereas 800 μL of EBM-2 with recombinant human
vascular endothelial growth factor (50 μg/L; Peprotech) was placed in
the lower chamber. EPCs were allowed to migrate in a humidified
incubator at 37°C for 24 h. The cells on the membrane’s lower side were
fixed with 4% paraformaldehyde and stained with hematoxylin.
Non-
migrated cells were removed by gently wiping the membrane’s upper
surface with a cotton swab. The number of EPCs was counted at
a magnification of ×100, and the mean value of 5 random fields was
determined for each sample (24).

EPC tube formation assay. An EPC tube formation assay was performed by using an In Vitro Angiogenesis Assay Kit (Millipore).
ECMatrix gel solution was thawed at 4°C overnight, mixed with
ECMatrix diluent buffer, and placed in a 96-well plate at 37°C for 1 h
to allow the matrix solution to solidify. EPCs were harvested as with
trypsin/EDTA, and then 1 × 10<sup>5</sup> EPCs were plated on a matrix solution
with EGM-2 medium and incubated at 37°C for 4 h. Tube formation
was inspected under an inverted light microscope (×100 magnification).
Tube formation was counted in 5 random fields with the Image-Pro Plus
(Media Cybernetics) (23).

Re-endothelialization assay. Endothelial recovery was evaluated
by staining denuded areas with Evans blue dye (Sigma). The blue-
stained and total luminal areas were determined respectively, and
the re-endothelialized area was expressed as a percentage of the total
surface (% area) (25).

Western blot. Aliquots (40 μg) of protein extracts were separated by
SDS-PAGE and then transferred onto an Immobilon-P membrane
(Millipore). After blocking, the blots were incubated with various
primary antibodies followed by horse radish peroxidase–labeled sec-
dary antibodies. The protein bands were detected with a SuperSignal
West Pico Chemiluminescent kit (Thermo). The primary antibodies used
were as follows: anti-AMP-activated protein kinase α (anti-AMPKα),
anti-endothelial nitric oxide synthase (anti-eNOS), anti-phospho-
AMPKα (Thr172), and anti-phospho-eNOS (Ser1177) (all from Cell
Signaling Technology).

Statistical analysis. Data (mean ± SD) were analyzed by using
GraphPad Prism (version 5.0; GraphPad Software) and SPSS (version
17.0; IBM SPSS Statistics, IBM Corporation). All variables were tested
by Shapiro-Wilk test for normal distribution. Differences were compared
by using 1-way ANOVA or Kruskal-Wallis H test (when variances were
unequal). Pairwise multiple comparisons were made with the Bonferroni
method to detect significant differences between groups when the H test
was significant. P < 0.05 was considered significant.
Results

Body weight and food intake. The body weight of diabetic apoE−/− mice (17.6 ± 3.02 g) was lower compared with nondiabetic controls (25.3 ± 2.18 g; P < 0.05), and the daily food intake of diabetic animals (5.45 ± 0.78 g) was higher than their nondiabetic counterparts (3.07 ± 0.52 g; P < 0.01). Supplementation with C3G did not cause significant differences in body weight (17.8 ± 2.36 g) and food intake (5.14 ± 0.82 g). The amount of C3G consumed by the diabetic mice was 10.3 ± 1.64 mg/d.

Serum glucose and lipid concentrations. The serum total and LDL-cholesterol and glucose concentrations were higher in diabetic apoE−/− mice than in nondiabetic controls (P < 0.05; Table 1). Supplementation with C3G did not cause significant changes in blood glucose or serum cholesterol relative to untreated diabetic mice.

Endothelium-dependent relaxation response. The sensitivity and maximal response of thoracic arteries to acetylcholine in diabetic apoE−/− mice were markedly reduced compared with that in nondiabetic apoE−/− mice. Diabetic apoE−/− mice treated with C3G displayed a significant improvement in acetylcholine-elicted relaxation compared with diabetic apoE−/− mice (Fig. 1A). There was no obvious differences in aortic smooth muscle relaxation to sodium nitroprusside among the 3 groups (Fig. 1B).

Number of circulating EPCs. The percentage of circulating EPCs (Sca-1/Fk-1 double-positive cells) in diabetic apoE−/− mice was dramatically lowered, from 10.3 ± 1.09% to 0.60 ± 0.33% in mononuclear cells (P < 0.01). The percentage of circulating EPCs (9.10 ± 2.57%) was much higher in C3G-treated diabetic apoE−/− mice compared with untreated diabetic mice (P < 0.01).

EPC functions and vascular repair. In vitro analysis of EPC adhesion, migration, and angiogenesis showed significant impairment of EPC functions in diabetic apoE−/− mice compared with nondiabetic mice. Supplementation with C3G significantly increased EPC adhesion (P < 0.01; Fig. 2A), promoted EPC migration (P < 0.01; Fig. 2B), and enhanced angiogenesis (P < 0.05; Fig. 2C). Compared with nondiabetic apoE−/− mice, re-endothelialization was impaired in diabetic apoE−/− mice. On the contrary, C3G supplementation significantly reversed diabetes-induced impairment in re-endothelialization (P < 0.05; Fig. 2D).

Endothelial CD31 level. CD31 staining was weak in the intima of diabetic mice, whereas nondiabetic mice demonstrated strong staining. C3G intervention restored the expression to the level of staining of nondiabetic controls (Fig. 3A). Optical density analysis of immunohistochemical staining provided quantification of the changes in CD31 expression (Fig. 3B).

AMPK activation and eNOS expression. Compared with EPCs isolated from nondiabetic apoE−/− mice, EPCs from diabetic mice showed a significant decrease in the phosphorylation levels of both AMPK (P < 0.05; Fig. 4A) and eNOS protein (P < 0.05; Fig. 4B), which was significantly reversed by C3G treatment.

Lesion area of atherosclerosis in aortic sinus. The mean atherosclerotic lesion area in the aortic sinus in the diabetic mice was 2.5-fold greater than in nondiabetic apoE−/− mice (P < 0.001). Supplementation with C3G significantly reduced the mean lesion area versus that in untreated diabetic mice (P < 0.05 vs. untreated diabetic mice; Supplemental Fig. 1A, B).

Discussion

To our knowledge, the present study is the first in vivo characterization showing that anthocyanin C3G supplementation potentially increased the number of circulating EPCs and largely improved the impaired EPC functions, including adhesion, migration, and tube formation capacities, under diabetic conditions.

TABLE 1 Serum glucose and lipid concentrations in nondiabetic apoE−/− mice, diabetic apoE−/− mice, and diabetic apoE−/− mice fed C3Ga

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>D</th>
<th>D + C3G</th>
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<tbody>
<tr>
<td>Glucose</td>
<td>5.36 ± 2.05b</td>
<td>10.1 ± 5.99a</td>
<td>11.1 ± 6.07a</td>
</tr>
<tr>
<td>TC</td>
<td>11.3 ± 3.12b</td>
<td>32.4 ± 13.2a</td>
<td>32.8 ± 4.59a</td>
</tr>
<tr>
<td>TgS</td>
<td>1.56 ± 0.62</td>
<td>1.53 ± 1.12</td>
<td>1.77 ± 1.50</td>
</tr>
<tr>
<td>HDL-C</td>
<td>0.58 ± 0.15</td>
<td>0.57 ± 0.18</td>
<td>0.65 ± 0.19</td>
</tr>
<tr>
<td>LDL-C</td>
<td>1.25 ± 0.48b</td>
<td>6.27 ± 3.26a</td>
<td>5.47 ± 1.50a</td>
</tr>
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1 Values are means ± SDs, n = 9–13. Labeled means in a row without a common letter differ, P < 0.05. apoE−/−, apolipoprotein E-deficient; C3G, cyanidin-3-O-β-glucoside; D, diabetic; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; ND, nondiabetic control; TC, total cholesterol.
Correction of all these abnormalities by C3G is associated with the restoration of the activities of phosphorylated (p-)AMPK and p-eNOS and an increase in vascular repair in diabetic animals. Finally, C3G intake improves the deterioration in endothelium-dependent relaxation and prevents the acceleration of atherosclerosis in diabetic apoE<sup>−/−</sup> mice. Thus, our current findings indicate direct beneficial effects of anthocyanin C3G on EPCs and that it ameliorated diabetes-induced atherogenesis.

Many studies have identified that the intake of anthocyanin-rich food significantly improved the high-fat-diet-induced features of metabolic syndrome in animal models (26–28). Furthermore, purified anthocyanins from berries are more effective in preventing increased obesity (28). However, the beneficial effects of purified anthocyanin on diabetes-accelerated atherogenesis have not been investigated previously. We observed that supplementation of C3G for 6 wk strongly ameliorated the enhanced atherosclerotic plaque volume in diabetic apoE<sup>−/−</sup> mice. The effect of C3G on atherogenesis was independent of any significant change in the large increases in hyperglycemia induced by streptozotocin injection. This suggests that the effect of C3G on atherogenesis is not related to any metabolic influence or alteration in severity of diabetes.

Diabetes mellitus contributes to endothelial cell injury and dysfunction, leading to the development of diabetes-related vascular complications (29). Here we observed that the endothelium-dependent relaxation response to acetylcholine was markedly impaired in apoE<sup>−/−</sup> mice injected with streptozotocin compared with the non-streptozotocin-injected controls. C3G treatment robustly prevented the damage by endothelium-dependent vasodilatation associated with diabetes in apoE<sup>−/−</sup> mice. The effects of C3G were likely exerted directly on endothelial cells because smooth muscle relaxation responses to sodium nitroprusside were unaffected.

EPCs, immature cells capable of differentiating into mature endothelial cells, play a critical role in maintaining endothelial function (30). Dysfunction of EPCs potently initiates and accelerates the development of diabetic vascular disease due to an insufficient endothelial cell repair (9,31). Both type 1 and type 2 diabetes mellitus have been associated with low concentrations and poor function of circulating EPCs and causally associated with diabetes-induced impairment in vasculogenesis (7,11). This stipulation is verified by the current study, which showed that diabetic apoE<sup>−/−</sup> mice had decreased numbers of circulating EPCs, reduced adhesion ability and Matrigel tube formation (Millipore), and attenuated ex vivo angiogenesis compared with nondiabetic apoE<sup>−/−</sup> mice. C3G feeding elicited significant EPC-modulating effects, as evidenced by increased mobilization of EPCs into the circulation, incorporation of EPCs into injured blood vessel wall, and augmentation of endothelial repair in diabetic mice. Therefore, C3G appears to exert its protective effects against diabetes-induced impairment in re-endothelialization through its pleiotropic actions on almost all of the major steps involved in EPC-mediated neovascularization. The restoration of EPC number and function after C3G treatment was paralleled by increased concentrations of endothelial p-AMPK and p-eNOS.

Our study did have several limitations. This study involved the administration of an anthocyanin compound, C3G, but not natural berries or berry powder, which would have been more...
applicable to the clinical progression of atherosclerosis. Another issue was the amount of C3G consumed. Daily C3G intake, 10.3 mg/d per diabetic mouse, was higher than in previous reports, which may have been due to the greater food consumption of diabetic animals than nondiabetic mice. Finally, we only used a single dose of anthocyanin compound, which cannot provide a dose-dependent response of C3G on endothelial repair and angiogenesis in diabetic apoE<sup>−/−</sup> mice.

In summary, our study indicates that the greater effect of the anthocyanin in the diabetic apoE<sup>−/−</sup> mice is likely related to preventing the deterioration EPC function and endothelial function that occur in the response to the diabetic milieu. Further EPC-based translational studies of anthocyanin intervention are needed to provide a novel and valid alternative in diabetic patients with advanced vascular complications.

Acknowledgments

Y.Z. conducted the research; Y.Z., X.W., Y.W., and Y.L. analyzed the data; and M.X. designed the experiments and prepared the manuscript. All authors read and approved the final manuscript.

Literature Cited


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