Anthocyanin increases adiponectin secretion and protects against diabetes-related endothelial dysfunction

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1Guangdong Provincial Key Laboratory of Food, Nutrition and Health, Guangzhou, Guangdong Province, China; and 2Department of Nutrition, School of Public Health, Sun Yat-sen University (Northern Campus), Guangzhou, Guangdong Province, China

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Liu Y, Li D, Zhang Y, Sun R, Xia M. Anthocyanin increases adiponectin secretion and protects against diabetes-related endothelial dysfunction. Am J Physiol Endocrinol Metab 306: E975–E988, 2014. First published March 4, 2014; doi:10.1152/ajpendo.00699.2013.— Adiponectin is an adipose tissue-secreted adipokine with beneficial effects on the cardiovascular system. In this study, we evaluated a potential role for adiponectin in the protective effects of anthocyanin on diabetes-related endothelial dysfunction. We treated db/db mice on a normal diet with anthocyanin cyanidin-3-O-β-glucoside (C3G; 2 g/kg diet) for 8 wk. Endothelium-dependent and -independent relaxations of the aorta were then evaluated. Adiponectin expression and secretion were also measured. C3G treatment restores endothelium-dependent relaxation of the aorta in db/db mice, whereas diabetic mice treated with an anti-adiponectin antibody do not respond. C3G treatment induces adiponectin expression and secretion in cultured 3T3 adipocytes through transcription factor forkhead box O1 (FoxO1). Silencing FoxO1 expression prevented C3G-stimulated induction of adiponectin expression. In contrast, overexpression of FoxO1-ADA promoted adiponectin expression in adipocytes. C3G activates FoxO1 by increasing its deacetylation via silent mating type information regulation 2 homolog 1 (Sirt1). Furthermore, purified anthocyanin supplementation significantly improved flow-mediated dilation (FMD) and increased serum adiponectin concentrations in patients with type 2 diabetes. Changes in adiponectin concentrations positively correlated with FMD in the anthocyanin group. Mechanistically, adiponectin activates cAMP-PKA-eNOS signaling pathways in human aortic endothelial cells, increasing endothelial nitric oxide bioavailability. These results demonstrate that adipocyte-derived adiponectin is required for anthocyanin C3G-mediated improvement of endothelial function in diabetes.

Anthocyanin; adiponectin; diabetes; endothelial function

OBESITY AND DIABETES are major risk factors for the initiation of vascular dysfunction and cardiovascular disease (21, 22). Adipose tissue is now recognized as an important metabolic and endocrine organ in the regulation of glucose metabolism. Dysregulation of adipose tissue contributes to the development of insulin resistance and the vascular complications of diabetes (12). Adiponectin is an adipocyte-derived plasma protein with both antiatherogenic and insulin-sensitizing properties, and lower plasma concentrations of adiponectin are closely correlated with obesity, insulin resistance, and diabetes mellitus (15, 26, 30, 44). In addition, hypoadiponectinemia is strongly associated with impaired endothelium-dependent vasorelaxation in both diabetic and nondiabetic human subjects (25, 33). Thus, modulating adiponectin levels in diabetic conditions would be beneficial for maintaining vascular function (35, 46).

Anthocyanins, as a group of flavonoids, are most abundant in various colorful fruits, vegetables, red wine, and grains (13, 16, 38, 45). Epidemiological studies have demonstrated that a higher intake of anthocyanins is consistently associated with a significantly lower risk of type 2 diabetes (37, 43) and cardiovascular disease (5). Many studies have focused on cyanidin-3-O-β-glucoside (C3G), the best known and most investigated anthocyanin (13, 16, 45). Our previous studies have shown that dietary supplementation with C3G enhanced endothelium-dependent vasorelaxation and inhibited atherogenesis in hypercholesterolemic apoE−/− mice (41); the underlying mechanism may be associated with the induction of oxysterol efflux from endothelial cells by adiponectin (42). Furthermore, we also reported that C3G intake significantly improved insulin sensitivity in both diet-induced and genetic animal models of type 2 diabetes (11); however, both the impact of C3G on vascular function in diabetes and the molecular mechanism that confers vascular protection are poorly understood.

The present study was designed to test the hypothesis that adipocyte-derived adiponectin plays an essential role in the amelioration of diabetes-induced endothelial dysfunction by anthocyanin C3G.

MATERIALS AND METHODS

Cell culture. 3T3-L1 adipocytes (ATCC, Manassas, VA) were maintained in high-glucose DMEM (HG-DMEM; GIBCO, Gaithersburg, MD) supplemented with 10% bovine serum and penicillin-streptomycin (Invitrogen). For differentiation (6), cells were incubated in HG-DMEM with 10% fetal bovine serum (FBS, Thermo Scientific) for 48 h after confluence. Cells were then cultured in differentiation medium I (HG-DMEM, 10% FBS, 1 μg/ml insulin, 0.25 μg/ml dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine) for 4 days, followed by differentiation medium II (HG-DMEM, 10% FBS, 1 μg/ml insulin) for 48 h. Prior to experiments, cells were cultured overnight in low-glucose DMEM (LG-DMEM, Invitrogen) with 10% FBS. All experiments were performed in LG-DMEM.

Fresh wild-type rat primary adipocytes and human primary subcutaneous adipocytes were supplied by Applied Biological Materials (Richmond, BC, Canada). Upon arrival, the cells were plated according to the manufacturer’s instructions. Cells were exposed to incubation medium at 37°C with 5% CO2.

Human aortic endothelial cells (HAECs) were purchased from Cell Applications (San Diego, CA) and cultured in M199 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), heparin (90 μg/ml), and endothelial cell growth supplement (20 μg/ml) at 37°C in humidified 5% CO2 (17). Cells were switched to normal or high-glucose (HG) culture medium for the indicated time period. HG conditions were achieved by the addition of 30 mM
glucose, with normal glucose (NG, 5 mM glucose + 25 mM mannitol) used as osmotic control.

Experimental animals. Eight-week-old male db/db mice (Jackson Laboratory, Bar Harbor, ME) were housed under standard conditions with a 12:12-h light-dark cycle and free access to food and water. Mice were fed a standard purified mouse diet (AIN-93, control group) or AIN-93 plus C3G (2 g/kg diet, C3G group) ad libitum for 8 wk. Some C3G-fed mice also received intravenous injections of anti-
adiponectin antibody (0.5 µg/g body wt) three times a week during the last 4 wk of the intervention (C3G-anti-Apn group). Each group contained eight mice. Anthocyanin C3G was mixed to homogeneity during the preparation of these diets. The temperature of the chow diet never exceeded 30°C, and the food was kept away from light whenever possible to ensure the stability of C3G. Stock C3G and all chows were stored in the dark at −40°C. Mice received fresh chow every third day, and food consumption and body weight were monitored weekly. Food intake and body weights did not differ between the two groups during the study. All experiments were approved by the Institutional Animal Care and Use Committee and were consistent with the Guide for the Care and Use of Laboratory Animals published by the Sun Yat-sen University.

Vascular reactivity. After mice were euthanized, thoracic aorta were removed and placed in oxygenated ice-cold Krebs solution. Changes in isometric tone of the aortic rings were recorded with a DMT wire myograph (AD Instruments Pty, Bella Vista, NSW, Australia). Rings were stretched to an optimal baseline tension and then allowed to equilibrate for 60 min before the experiment commenced. Rings were initially contracted with 60 mM KCl and rinsed in Krebs solution. After several washouts, phenylephrine (1 µM) was used to produce a steady contraction, and acetylcholine (ACh, an endothelium-dependent vasodilator, 10−9 to 10−5 M) was added cumulatively to induce endothelium-dependent relaxation. Endothelium-independent relaxation was stimulated in aortic rings with sodium nitroprusside (SNP, an endothelium-independent nitric oxide-releasing agent, 10−9 to 10−5 M), and the vascular relaxation was recorded (41).

Adenoviral vectors and infection. Adenoviruses encoding hemagglutinin (HA)-tagged constitutively nuclear (ADA) or dominant negative (Δ256) Foxo1 were constructed as previously described or obtained from Addgene (Cambridge, MA), respectively (40). Foxo1-ADA has mutated Akt phosphorylation sites (Thr32/36, Ser256, Asp, and Ser1177), and Foxo1-Δ256 lacks the transactivation domain and competes with endogenous Foxo1. Recombinant viruses containing constructs were amplified in HEK-293 cells and isolated by cesium chloride density-gradient centrifugation. Viruses were collected and desalted, and titers were measured using Adeno-XTM Rapid titer (BD Bioscience, San Jose, CA) according to the manufacturer’s instructions.

Quantitative real-time polymerase chain reaction. Cells were harvested in TRIzol (Invitrogen), and total RNA was isolated according to the manufacturer’s instructions. Quantitative (q)RT-PCR was performed as previously described (9), using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) on the ABI 7500 DNA Sequencing Detection System with standard fluorescent chemistries. cDNA products were quantified using LightCycler software (ΔΔCT method) and normalized to housekeeping gene NADPH.

Small interfering RNA. For experiments with RNAi, 3′-UTR-L1 adipocytes were transfected using Lipofectamine 2000 with 100 nM ON-TARGET plus SMARTpool for mouse Foxo1 and Sirt1 (Dharmacon, Thermo Fisher Scientific). Scrambled oligonucleotides (ON-TARGET plus siCONTROL Non-Targeting Pool) were used as controls. After 24 h, cells were washed with PBS, treated with C3G for 24 h, and harvested for further analysis. Knock-down efficiency of each gene was determined by immunoblotting.

**Acetylation assay.** Cells were washed with ice-cold PBS and then cold lysis buffer (25 mM Tris-HCl, pH 7.9, 5 mM MgCl2, 10% glycerol, 100 mM KCl, 1% NP-40, 0.3 mM dithiothreitol, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 50 mM sodium fluoride) containing a protease inhibitor cocktail (Calbiochem) was added. For acetylation studies, 5 mM nicotinamide and 1 mM sodium butyrate were added to the buffer. Cells were then transferred to an Eppendorf tube, left on ice for 15 min, and centrifuged at 10,000 g for 10 min. The supernatant was collected and stored at −80°C. To evaluate Foxo1 acetylation, 1,000 µg of total protein was first immunoprecipitated from cells with anti-Foxo1 antibody and then immunoblotted for acetyl-Foxo1 (Ac-Foxo1). Bands were detected using ultrasensitive horseradish peroxidase chemiluminescence (Pierce, Rockford, IL).

**Adiponectin secretion assay.** Cultured media were immunoprecipitated by using Catch and Release v. 2.0 Catch-and-Release reversible immunoprecipitation system (Upstate, Charlottesville, VA) for immunocomplex pull-down. After treatment, the supernatants of culture medium from each well (500 µl) were collected, and protein concentration was measured. The supernatant was then added to the antibody capture affinity ligand and the specific antibody against adiponectin (anti-Apn, Chemicon International) and incubated in a Catch and Release spin column at room temperature for 1 h under continuous shaking. The column was then washed three times with the wash buffer (10% Nomidet P-40, 2.5% deoxycholic acid, 150 mM imidazole, pH 7.4). The immunoprecipitate was then eluted with Tris-based immunoprecipitation elution buffer. Immunoprecipitates containing equivalent amounts of total protein were subjected to immunoblotting using anti-Apn antibody. The β-actin obtained by running lysates of the adipocytes from the same well was used as the loading control and this represents a comparable cell number of 1×10⁶/well.

**Immunoprecipitation.** Approximately 500 mg of protein from cultured cells was used for immunoprecipitation. Forty microliters of protein A-Sepharose suspended in lysis buffer was used for preclarifying the sample and immunoprecipitation after conjugating the beads with 3–5 µg of the specific antibody. The resulting immunoprecipitate was boiled with Laemmli sample buffer and used for immunoblotting.

**Western blot.** Adipocyte lysates or mouse adipose tissue homogenates, collected with RIPA buffer (50 mM Tris, 150 mM sodium chloride, 1% NP-40, 12 mM sodium deoxycholate, 3.5 mM SDS, pH 7.4) and protease inhibitor cocktail (Roche Diagnostics), were electrophoresed through 8.0%-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). Nonspecific binding sites were blocked with 1% nonfat milk in Tris-buffered saline with 0.05% Tween 20 detergent (TWEEN-20TBS). The blots were then incubated overnight at 4°C with the primary antibodies including anti-adiponectin (Chemicon International), anti-phospho-endothelial nitric oxide synthase (eNOS) at Ser177, anti-eNOS, anti-acetyl-lysine of Foxo1, anti-phospho-Thr³⁴ of Foxo1, anti-Foxo1, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Anti-β-actin was used as an internal immunoblot control to confirm equal protein loading. All of the other antibodies were obtained from Cell Signaling Technology.

**Fig. 1.** Adiponectin mediates cyanidin-3-O-β-glucoside (C3G)-induced improvement of vascular reactivity in db/db mice. A: anti-adiponectin antibody (anti-Apn) abolished the effect of C3G to improve endothelium-dependent relaxation in aorta of db/db mice. *P < 0.05 vs. corresponding Control or C3G-anti-Apn group. B: cGMP levels in aorta of db/db mice. C: phosphorylation level of endothelial NO synthase (eNOS) Ser¹⁷⁷ in aorta from db/db mice was determined by immunoblotting and expressed as fold of control. D: adiponectin secretion in culture medium of subcutaneous, visceral, and perivascular adipose tissue isolated from db/db mice of Control and C3G group. Results are means ± SD of 4 experiments. *P < 0.05 vs. Control. E: adiponectin secretion in culture medium of subcutaneous adipose tissue isolated from db/db mice of Control, C3G, and C3G-anti-Apn group. Results are means ± SD of 4 experiments. *P < 0.05 vs. Control. F: serum adiponectin level was measured by an ELISA-based method. Results are means ± SD of 3 independent experiments. *P < 0.05 vs. Control or C3G-anti-Apn; n = 8 per group. G: representative blot (top) showing distribution of adiponectin oligomers (HMW, MMW, and LMW) in db/db mice from Control and C3G groups. Quantification of Western blot data (bottom). Results are means ± SD; n = 4 per group. *P < 0.05 vs. corresponding Control.
Bands were visualized with an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

Chromatin immunoprecipitation. Chromatin was extracted from 3T3-L1 adipocytes on day 9 after differentiation using the EZ-ChIP kit (Upstate, Billerica, MA). Adipocytes were treated with C3G for 24 h prior to cross-linking for 10 min with 1% formaldehyde. Cells were then lysed and sonicated three times for 20 s using a sonic dismembrator (Fisher Scientific). Lysates were precleared with protein A-agarose beads (Millipore). FKHR or PPARγ/H9253 antibody (Santa Cruz Biotechnology) was applied. DNA was released from protein-DNA complexes by proteinase K digestion and then subjected to quantitative real-time PCR for the adiponectin response elements for PPARγ (PPRE) and Foxo1 using the Power SYBR Green Kit (Applied Biosystem). Chromatin immunoprecipitation (ChIP)-qPCR data were normalized to input samples for the amount of chromatin and for immunoprecipitation efficiency by normal IgG controls (9).

Sirt1 deacetylase activity. Sirt1 deacetylase activity was measured using the fluorometric Sirt1 Assay Kit (Sigma Aldrich, CS1040) according to the manufacturer’s instructions. Fluorescence intensity at 444 nm (excitation 355 nm) was recorded and normalized to micrograms of protein. Values are represented as fold of control.

Study subjects and design. A total of 58 adult subjects with type 2 diabetes mellitus aged 56–67 yr were recruited into this clinical trial from physical examination centers at a single hospital in Guangzhou, Guangdong, China, between November 2008 and December 2010. We excluded patients who were pregnant or recently diagnosed with diabetes mellitus or who had coronary artery disease or other known chronic diseases. The study was approved by the Ethics Committee of Sun Yat-sen University and was conducted in accordance with the Declaration of Helsinki. Participation was voluntary, and each participant provided written informed consent. For the intervention study, eligible participants were randomized in a double-blind, placebo-controlled, parallel, 12-wk trial and assigned to either the anthocyanin group (n = 29) or the placebo group (n = 29). During the trial period, the participants were instructed to consume two anthocyanin capsules or placebo capsules twice daily (30 min after breakfast and supper).
The anthocyanin capsules provided a total daily intake of 320 mg of anthocyanins. They were also asked to maintain their habitual diet and lifestyle.

Blood sampling and analysis. Serum total cholesterol was determined with the Cholesterol CHOP-PAP kit (12016630 122, Roche/Hitachi), and HDL-cholesterol with an enzymatic HDL-Cholesterol kit (Biomed Laboradiostik). Triglycerides in serum were quantified using Triglycerides GPO-PAP kit (12016648 122, Roche/Hitachi). LDL subsequently was calculated using the Friedewald formula (8). Insulin was analyzed using a time-resolved immunofluorometric assay (AutoDELFIA Insulin kit, PerkinElmer), and free fatty acid (FFA) by a commercially available kit (Wako Chemicals). Serum glucose levels were measured using the glucose oxidation method, and glycosylated hemoglobin A1c (HbA1c) by high-pressure liquid chromatography. C-peptide was analyzed using a commercially available ELISA kit (Millipore).

Serum adiponectin measurement. Mice were fasted overnight, and venous blood samples were collected by retroorbital puncture. Serum levels of mouse adiponectin were measured using a commercially available ELISA kit (AdipoGen, Seoul, Korea). The distribution of adiponectin oligomers in serum was analyzed on the HiLoad 16/60 Superdex 200 prep grade column (Sigma-Aldrich) as previously described (7). Two hundred microliters of serum samples was fractionated at 0.5 ml/min using PBS containing 5 mM DTT as elution buffer (n = 4 per group). Fractions (0.5 ml) were collected, and the concentration of adiponectin oligomers was measured by ELISA kit. The oligomeric composition was then assessed by immunoblotting. Human serum adiponectin concentrations were measured in stored frozen baseline serum using a commercially available competitive ELISA kit according to the manufacturer’s instructions. The assay used standards in the range of 0.001 to 1 μg/ml. The intra-assay and interassay coefficients of variation were 4% and 3%, respectively, as previously described (39).

Assessment of endothelium-dependent relaxation. Endothelial function was assessed by blood flow, flow-mediated dilatation (FMD), and endothelium-independent glyceryltriminitrate-induced dilation (GTND) measurements taken noninvasively in the right brachial artery with a high-frequency ultrasound scanning machine (Sonos 4500, Phillips Medical Systems) and a high-resolution (7.5 MHz) linear array transducer as previously described (47).

Statistical analysis. Continuous data are expressed as means ± SD. Comparison between groups was analyzed using one-way analysis of variance followed by the Student-Newman-Keuls test. P values of <0.05 were accepted to indicate statistically significant differences. Nonquantitative results are representative of at least three independent experiments. Pearson correlation coefficients (r) were used to determine the association between changes in serum adiponectin concentrations and changes in FMD in the 12-wk study.

RESULTS

C3G increases serum adiponectin concentrations and improves endothelial function in diabetic mice. We first tested the endothelium-dependent arterial relaxation to endothelium-dependent Ach in mice by using isometric tension studies. Compared with untreated db/db mice, C3G treatment caused a remarkable augmentation in the aortic endothelium-dependent vasorelaxation response to Ach, indicating a significant improvement of endothelial function (Fig. 1A). NO bioavailability is the most important factor for maintaining vascular homeostasis and integrity. Therefore, we next assessed cGMP level as the marker of NO bioavailability. The cGMP levels in aorta were significantly increased in C3G-treated mice compared with untreated control db/db mice (Fig. 1B). To obtain more insight into the mechanisms for preservation of NO bioavailability by C3G, we measured eNOS expression. C3G increased eNOS phosphorylation at Ser1177 in the aorta of db/db mice but did not affect total eNOS protein expression.
(Fig. 1C). The improved endothelial-dependent relaxation and eNOS signaling after in vivo C3G treatment was markedly prevented by in vivo injection of anti-Apn antibody (Fig. 1, A–C). Furthermore, C3G treatment stimulated a significant adiponectin release in subcutaneous fat (Fig. 1D), which was significantly prevented in the presence of anti-Apn antibody (Fig. 1E). C3G treatment also elevated the serum levels of adiponectin in db/db mice (Fig. 1F). The analysis of oligomeric complex distribution of adiponectin revealed an increase in three major molecular mass species corresponding to low-molecular-weight (LMW), medium-molecular-weight (MMW), and high-molecular-weight (HMW) adiponectin oligomers (~60, ~150, and ~250 kDa, respectively; Fig. 1G). In particular, HMW oligomers were ~2.5-fold higher in the C3G group than in the control group, MMW oligomers were 1.7-fold higher in the C3G group than in the control group, and LMW oligomers were 1.5-fold higher in the C3G group than in the control group.

C3G increases adiponectin transcription and secretion in adipocytes. To investigate the effects of C3G on the regulation of adiponectin expression in adipocytes, we incubated 3T3-L1 murine adipocytes with C3G (25 μM) for 2–24 h and examined the effects on adiponectin mRNA expression. C3G treatment caused a time-dependent increase in the expression of adiponectin mRNA as determined by quantitative RT-PCR (Fig. 2A). The levels of adiponectin mRNA increased as early as 4 h after the addition of C3G, and increased ~2.4-fold over the controls by 12 h. The adiponectin transcript levels remained high throughout the 24-h treatment period. Next, we incubated adipocytes with various concentrations of C3G (12.5, 25, 50 μM) for 24 h and found that adiponectin mRNA expression was enhanced in a dose-dependent fashion (Fig. 2B). We measured luciferase activity driven by the proximal 1,460 bp of the murine adiponectin promoter, which contains most of the previously identified sites that regulate adiponectin transcription (29). C3G treatment caused a concentration-dependent increase in adiponectin promoter activity (Fig. 2C). C3G did not affect the half-life of the endogenous mRNA measured after actinomycin D treatment of cells (Fig. 2D). To determine the effect of C3G on adiponectin secretion from adipocytes, we then performed immunoprecipitation on cell medium, followed by Western blots to detect the secretion of adiponectin protein. Treatment with C3G resulted in a dose-dependent induction of adipocyte adiponectin secretion into the medium compared with control, untreated 3T3-L1 adipocytes (Fig. 2E).

To determine whether the stimulatory effect of C3G on adipocyte adiponectin expression in vitro is relevant to humans and to rodent models, we treated rat adipocytes and primary human adipocytes with C3G for 24 h. In accord with the observations in 3T3-L1 adipocytes results above, we observed significantly greater adiponectin mRNA expression (Fig. 3, A and B) and secretion (Fig. 3, C and D) in C3G-treated cells vs. untreated control cells in both species tested (rat and human). Thus, we confirmed that our key finding is applicable to humans and rodent models.

C3G upregulates adiponectin transcription via Foxo1. Most physiological regulation of adiponectin gene transcription is attributable to transcription factors Foxo1 and PPARγ (18, 29). We therefore examined the potential role of these two regulators in C3G-mediated induction of adiponectin transcription. Inhibition of PPARγ with antagonist GW-9662 exerted little influence on adiponectin expression and secretion (Fig. 4, A and B). However, pharmacological inhibition of Foxo1 with AS-1708727 markedly prevented C3G-mediated induction of adiponectin mRNA expression (Fig. 5A) and secretion (Fig. 5B). Furthermore, adiponectin mRNA levels (Fig. 5C) and secretion (Fig. 5D) in C3G-treated adipocytes were similar to those observed in vehicle-treated cells after genetic inhibition of Foxo1 by transfecting cells with Foxo1 siRNA. These data suggest that Foxo1 is required for C3G-mediated upregulation of adiponectin. To further investigate the essential role of Foxo1, we analyzed the ability of gain- and loss-of-function Foxo1 mutants to affect C3G-induced adiponectin expression. Transduction of 3T3-L1 adipocytes with adenoviral vectors encoding FOXO1-D256 (a dominant negative form of Foxo1) increased the phosphorylation of Thr24, which is responsible for FOXO nuclear exclusion (24). In contrast, phosphorylation on a constitutively nuclear form of Foxo1 (Foxo1-ADA) on Thr24 was not detected (Fig. 5E). Furthermore, Foxo1-ADA transfection markedly increased adiponectin mRNA expression in the absence or presence of C3G. However, ectopic expression of the Foxo1-D256 mutant dramatically decreased adiponectin expression even in the presence of C3G (Fig. 5F). These data support the hypothesis that Foxo1 is necessary and
sufficient for the induction of adiponectin gene transcription by C3G.

**C3G induces deacetylation of Foxo1 and promotes Foxo1 assembly with C/EBPα to the adiponectin promoter.** Deacetylation of Foxo1 is generally associated with increased adiponectin transcription. We speculated that C3G increases adiponectin expression through by altering Foxo1 acetylation status and activity. Immunoprecipitation using an anti-acetyl-lys antibody followed by blotting with anti-Foxo1 antibody revealed that C3G treatment decreased Foxo1 acetylation as
early as 4 h after treatment, which became more evident throughout the 24-h treatment period (Fig. 6A). C3G also dose-dependently reduced Foxo1 acetylation (Fig. 6B). There was no change in Ser256 phosphorylation or total Foxo1 protein with C3G treatment. We further monitored whether C3G-mediated Foxo1 deacetylation influences Foxo1 transcriptional activity. We observed that C3G treatment robustly increased Foxo1 transcriptional activity in adipocytes (Fig. 6C).

We next measured Foxo1 occupancy in its two known sites of transcriptional activation by ChIP assays. As predicted by Foxo1 acetylation status, cells treated with C3G exhibited a dose-dependent increase in occupancy of the adiponectin promoter by endogenous Foxo1 (Fig. 6D). Because the interaction of C/EBPα with Foxo1 has been implicated in regulation of adiponectin transcription (33), we measured the association of C/EBPα with Foxo1 by coimmunoprecipitation. C3G treat-

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**Fig. 6.** C3G promotes Foxo1 deacetylation and induces Foxo1 binding to the adiponectin promoter. A: 3T3-L1 adipocytes were treated with C3G for the indicated time. Total protein lysates were then used for immunoprecipitation of Foxo1, and Foxo1 acetylation level was measured by Western blot. B and C: 3T3-L1 adipocytes were treated with different concentrations of C3G for 24 h. Foxo1 acetylation level (B) and Foxo1 transcriptional activity (C) were measured as indicated, respectively. Data are expressed as means ± SD from 3 independent experiments. *P < 0.05 vs. Control. D: ChIP assay showing Foxo1 recruitment to the adiponectin promoter following treatment with C3G. Data are means ± SD from 3 independent experiments. *P < 0.05, **P < 0.01 vs. Control. E: immunoprecipitation of 3T3-L1 adipocyte extracts treated overnight in the presence or absence of increasing concentrations of C3G and then incubated with antibodies against Foxo1 and immunoblotted for Foxo1 and C/EBPα. Data are means ± SD from 3 independent experiments. *P < 0.05, **P < 0.01 vs. Control.
ment resulted in a 3.5-fold enhancement of C/EBPβ binding to Foxo1 (Fig. 6E).

**C3G activates Foxo1 through Sirt1.** Because Sirt1 interacts with and deacetylates Foxo1 (4), we next evaluated the possible role of Sirt1 in the C3G-mediated deacetylation of Foxo1. C3G failed to decrease Foxo1 acetylation (Fig. 7A) or increase transcriptional activity (Fig. 7B) when Sirt1 expression was knocked down with specific siRNA. The lack of Sirt1 also compromised the induction of adiponectin expression by C3G (Fig. 7C). In parallel with reduced Foxo1 deacetylation, incubation with C3G increased Sirt1 deacetylase activity in adipocytes in a dose-dependent manner (Fig. 7D). These results indicate that Sirt1 is necessary for C3G to increase Foxo1-mediated adiponectin expression.

**Anthocyanin increases serum adiponectin levels and improves endothelial function in diabetic subjects.** To confirm the effect of anthocyanin on adiponectin production, we conducted a clinical study. Forty-eight consecutive patients with diabetes who met the inclusion criteria were enrolled in this clinical study. The patients were randomized to either the anthocyanin group or the placebo group. Baseline variables and treatment for the two groups are shown in Table 1. There were no differences in baseline clinical characteristics, hemodynamics, biochemical data, or medications. There was also no significant difference in the baseline serum level of adiponectin between the two groups. The serum adiponectin levels at 12 wk following anthocyanin administration were significantly increased in the anthocyanin group compared with baseline (Fig. 8A). Furthermore, there were significant increases in the FMD after the 12-wk anthocyanin supplementation (Table 2). There were no significant changes in serum adiponectin concentrations or FMD throughout the study in the placebo group. The changes in adiponectin concentration after anthocyanin intervention were found to be positively correlated with changes in FMD ($r = 0.661$, $P < 0.001$; Fig. 8B), this correlation was not observed in the placebo group ($r = 0.137$, $P = 0.469$; Fig. 8C).

**Table 1. Characteristics of the study population**

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<th>Characteristics</th>
<th>Placebo ($n = 29$)</th>
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<tr>
<td>Age, yr</td>
<td>$62.3 \pm 5.8$</td>
<td>$63.2 \pm 7.3$</td>
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<td>17/12</td>
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<tr>
<td>BMI, kg/m²</td>
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<td>$25.5 \pm 4.2$</td>
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<td>Systolic BP, mmHg</td>
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<td>$131 \pm 3$</td>
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<td>Diastolic BP, mmHg</td>
<td>$78 \pm 8$</td>
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<td>Fasting plasma glucose, mM</td>
<td>$7.8 \pm 1.5$</td>
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<td>HbA1c, %</td>
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<td>$3.14 \pm 0.37$</td>
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Values are means ± SD.
No significant mean differences in the brachial diameter, baseline and hyperemic blood flow, or GTND were observed between the two groups after the 12-wk intervention (Table 2). No such effect was evident in the placebo group. These results suggested that C3G supplementation led to an increase of the serum adiponectin level in diabetes population.

_Serum adiponectin activates cAMP-PKA-eNOS signaling in endothelial cells under hyperglycemic conditions._ Next, we performed in vitro studies using HAECs cultured in serum derived from control and anthocyanin-treated patients. Compared with quiescent HAECs, stimulation of HAECs with high glucose (HG) repressed the eNOS activity, which was reversed with adiponectin serum but not placebo serum (Fig. 9A). Adiponectin serum also restored the reduced cGMP production under hyperglycemic conditions (Fig. 9B). Notably, depletion of adiponectin from the serum using lysine-Sepharose reduced adiponectin serum-stimulated eNOS activation (Fig. 9C) and cGMP production (Fig. 9D). In addition, compared with quiescent HAECs, HG-stimulated HAECs showed a reduction in adenylyl cyclase enzyme activity (Fig. 10A), cAMP levels (Fig. 10B), and PKA activity (Fig. 10C) compared with quiescent HAECs. This was reversed by incubation with anthocyanin serum but not placebo serum (Fig. 10, A–C). Furthermore, pretreatment of HAECs with H89 or SQ-22536 almost totally abolished anthocyanin serum-mediated eNOS activation (Fig. 10D) and cGMP production (Fig. 10E).

**DISCUSSION**

The present study demonstrated a novel mechanism for anthocyanin C3G in the preservation of endothelial function under diabetic conditions. Supplementation with C3G resulted in a marked improvement in endothelium-dependent relaxation in mouse models of type 2 diabetes. C3G treatment preserved eNOS activity and NO availability and increased serum adiponectin levels in diabetic mice. In vitro findings showed that C3G upregulated adiponectin expression and secretion in adipocytes. The underlying molecular mechanism for C3G-induced adiponectin expression is mainly attributed to Sirt1-dependent Foxo1 acetylation. Finally, we demonstrated that chronic anthocyanin supplementation improves FMD in type 2 diabetic patients. Ex vivo studies showed that adipocyte-derived adiponectin preserved NO bioavailability in HAECs through a cAMP-PKA-dependent pathway under hyperglyce-

![Graph](https://example.com/graph.png)

**Table 2. Metabolic data and endothelial function of the study groups**

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Anthocyanin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>125 ± 6</td>
<td>126 ± 8</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>78 ± 8</td>
<td>77 ± 6</td>
</tr>
<tr>
<td>Fasting plasma glucose, mM</td>
<td>7.7 ± 1.5</td>
<td>7.5 ± 1.9</td>
</tr>
<tr>
<td>Hba1c, %</td>
<td>7.1 ± 0.8</td>
<td>7.3 ± 0.7</td>
</tr>
<tr>
<td>Serum insulin, pM</td>
<td>52 ± 23</td>
<td>54 ± 31</td>
</tr>
<tr>
<td>Serum C-peptide, nM</td>
<td>0.86 ± 0.29</td>
<td>0.84 ± 0.35</td>
</tr>
<tr>
<td>Serum FFA, mM</td>
<td>0.79 ± 0.24</td>
<td>0.75 ± 0.32</td>
</tr>
<tr>
<td>Total cholesterol, mM</td>
<td>4.9 ± 0.7</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>LDL-cholesterol, mM</td>
<td>3.12 ± 0.25</td>
<td>3.01 ± 0.33</td>
</tr>
<tr>
<td>HDL-cholesterol, mM</td>
<td>0.95 ± 0.07</td>
<td>0.92 ± 0.06</td>
</tr>
<tr>
<td>Triglycerides, mM</td>
<td>2.06 ± 0.67</td>
<td>2.02 ± 0.54</td>
</tr>
<tr>
<td>Brachial diameter, mm</td>
<td>4.02 ± 0.67</td>
<td>3.98 ± 0.72</td>
</tr>
<tr>
<td>Hyperemic blood flow, ml/min</td>
<td>352.4 ± 118.5</td>
<td>364.8 ± 128.4</td>
</tr>
<tr>
<td>FMD, %</td>
<td>7.84 ± 2.03</td>
<td>8.02 ± 1.97</td>
</tr>
<tr>
<td>GTND, %</td>
<td>17.6 ± 4.9</td>
<td>17.4 ± 6.2</td>
</tr>
</tbody>
</table>

Values are means ± SD. No significant differences were found for any variable between the 2 groups at baseline via unpaired Student *t*-test. FMD, flow-mediated dilation; GTND, glyceryltrinitrate-induced dilation. *P < 0.05, **P < 0.01 vs. baseline, assessed by paired Student’s *t*-tests.

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mic conditions. Thus, our present study indicates that adipose tissue can be an important target of anthocyanin by releasing vasoactive hormones, which may provide a novel rationale to its potential clinical impact on diabetic vascular disease protection.

Endothelial dysfunction, characterized by an imbalance between vasodilating and vasoconstricting substances, is associated with the occurrence and severity of vascular complications in diabetes mellitus (23). C3G treatment strongly improved endothelium-dependent relaxation in aorta and increased the amount of adiponectin released in \( \text{db/db} \) mice. The obligatory role of adipocyte-derived adiponectin in the vascular benefit of C3G is supported by the observation that a neutralizing anti-adiponectin antibody in vivo could prevent the improvement of vascular relaxation in \( \text{db/db} \) induced by C3G. Correct oligomerization of adiponectin protein was essential for biological activity. A previous study showed that the HMW complex was the most active form of adiponectin in lowering blood glucose levels in mice (27) and only the HMW form could protect endothelial cells from apoptosis. Our study demonstrated that C3G could increase production of the HMW form of adiponectin, demonstrating the elevated biologically active adiponectin. Thus, these results showed an indispensable role for adiponectin in the protective effect of anthocyanin C3G on diabetic vascular dysfunction. Increased oxidative stress and augmented inflammatory response play a pivotal role in the development of diabetes complications, both microvascular and cardiovascular. Anthocyanin C3G has been shown to possess high levels of antioxidant/anti-inflammatory activities (13, 16, 45), both of which may contribute to the beneficial impacts on the vascular function exerted by anthocyanin.

We further utilized 3T3-L1 adipocytes, which are widely used for studying adipogenesis and the biochemistry of adipocytes, to explore the effect of anthocyanin on adiponectin expression and secretion in vitro. In this study, we observed a positive, dose-dependent effect of C3G on adipocyte adiponectin expression and secretion. The stimulatory effect of C3G on adiponectin was present at all doses to a maximum at 50 \( \mu \)g/ml, which is a commonly used dose in the in vitro study. At that dose, C3G produced a nearly fourfold increase in adiponectin protein secretion into cultured medium vs. control cultured adipocytes. This finding was confirmed in primary adipocytes isolated from rat and human adipose tissue, demonstrating the in vivo relevance of these findings.

Most physiological regulation of adiponectin levels is attributable to transcriptional changes mediated by Foxo1 and PPAR\( \gamma \) (18, 29). We have demonstrated that anthocyanin causes decreased acetylation of Foxo1 without changing its level of phosphorylation. A previous study reported that Foxo1 interaction with C/EBP\( \alpha \) was enhanced by Sirt1, leading to
increased adiponectin transcription in 3T3-L1 cells (33).

We did see the increased binding of Foxo1 to the adiponectin promoter and enhancement of C/EBPβ binding to Foxo1 in response to C3G treatment. We observed that knockdown of Sirt1 impaired Foxo1 deacetylation, transcriptional activity, and adiponectin expression by C3G. Exposure to C3G resulted in a marked increase in Sirt1 deacetylase activity. Our results have added anthocyanin to the list of factors that are integrated by Foxo1 in determining its regulation of adipocyte metabolism and adiponectin secretion.

Although previous studies documented the stimulated effect of adiponectin expression by C3G in adipocytes (31, 36), our present study has extended these findings and uncovered the molecular mechanism through which C3G regulates adiponectin transcription. More important, we have elucidated that adipose tissue-derived adiponectin plays an obligatory role in anthocyanin-mediated alleviation of diabetes-induced vascular dysfunction.

The findings from animal studies are promising, and relatively low-dose anthocyanin interventions with patients clinically diagnosed with vascular diseases have been associated with significant reductions in ischemia (34), blood pressure (2), and lipid levels (10). However, human intervention and clinical studies are limited, with the application of purified anthocyanins on endothelial function relative to other subclasses of polyphenols in tertiary prevention being relatively unknown (14). Our previous 3-mo randomized, controlled trial showed that anthocyanin intake improved endothelium-dependent vasodilation in hypercholesterolemic individuals, effects that were thought to be mediated via the NO-cGMP pathway (47). We further explored whether pure anthocyanins would have sustained beneficial effects on endothelial function in patients with diabetes using a long-term intervention. In our cohort of diabetic patients who were administered anthocyanin for 12 wk, we observed that anthocyanin supplementation dramatically improved endothelial function, as determined by FMD measurement, whereas we observed no significant differences in GTND, suggesting that anthocyanins do not affect NO-independent vasodilation. These findings are consistent with previous reports showing that delphinidin, a monomer of anthocyanin, evokes vasorelaxation that is 89% endothelium dependent (1). Moreover, we found that increased serum adiponectin concentrations correlated with improved FMD in diabetic individuals treated with anthocyanin supplementation. Therefore, we suggest that the improvement in endothelium-dependent vasodilation by anthocyanins in individuals with diabetes occurs mainly through the induction of adiponectin secretion.

The adiponectin-induced improvement in endothelial-dependent relaxation can be mediated by the cAMP/PKA cascade. This conclusion is based on the observation that, in
cultured HAECs, adiponectin reversed the inhibition of eNOS activation and NO availability in response to HG. Further experiments demonstrated that adiponectin increased the cAMP content of HG- incubated HAECs through activation of adenylyl cyclase, thus leading to the induction of PKA-dependent eNOS phosphorylation.

In summary, the present study demonstrates that anthocyanin-activated, adipose tissue-derived adiponectin plays an obligatory role in anthocyanin-induced improvement of diabetes-related endothelial dysfunction.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


