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

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Abstract

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 \textit{db/db} mice on a normal diet with anthocyanin C3G (2 g/kg diet) for 8 weeks. Endothelium-dependent and independent relaxation of the aorta was then evaluated. Adiponectin expression and secretion were also measured. C3G treatment restores endothelium-dependent relaxation of the aorta in \textit{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 HAECs, increasing endothelial nitric oxide (NO) bioavailability. These results demonstrate that adipocyte-derived adiponectin is required for anthocyanin C3G-mediated improvement of endothelial function in diabetes.

Keywords: Anthocyanin, Adiponectin, Diabetes, Endothelial Function
Introduction

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 anti-atherogenic 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 anthocyanin (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 is 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 media 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 media 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 Inc. (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 Inc. (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, USA) were housed under standard conditions with a 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 wks. Some C3G-fed mice also received intravenous injections of anti-adiponectin antibody (0.5 μg/g bodyweight) three times a week during the last 4 wks of the intervention (C3G+anti-Apn group). Each group contained 8 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 killed, 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 Ltd, 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 (Thr24Ala, Ser256Asp, and Ser316Ala), and Foxo1-Δ256 lacks the transactivation domain and competes with endogenous Foxo1. Recombinant viruses containing constructs were amplified in HEK293 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 (qPCR).** Cells were harvested in TRIZOL (Invitrogen), and total RNA was isolated according to the manufacturer’s instructions. Quantitative RT-PCR was performed as previously described (9), using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) on the ABI 7500 DNA Sequence Detection System with standard fluorescent chemistries. cDNA products were quantified using LightCycler software ($\Delta\Delta$CT method) and normalized to housekeeping gene NADPH.
Small interfering RNA (siRNA). For experiments with RNAi, 3T3-L1 adipocytes were transfected using Lipofectamine 2000 with 100 nM ON-TARGET plus SMARTpool for mouse Foxo1 and Sirt1 (Dharmacon, Thermo Fisher Scientific Inc., CO, USA). Scrambled oligonucleotides (ONTARGET 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, then cold lysis buffer (25 mM Tris-HCl pH 7.9, 5 mM MgCl₂, 10% glycerol, 100 mM KCl, 1% NP40, 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, 1000 μg of total protein was first immunoprecipitated from cells with anti-Foxo1 antibody, 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® v2.0 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, Inc.) 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% Nonidet 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 pre-clearing the sample and immunoprecipitation after conjugating the beads with
3–5 mg of the specific antibody. The resulting immunoprecipitate was boiled with Laemmli
sample buffer and used for immunoblotting.

Western blot. Adipocytes 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, QC), were
electrophoresed through 8.0%–12% sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE) and transferred onto an immobilon-P polyvinylidene difluoride
membrane (Millipore Corp., Bedford, MA). Nonspecific binding sites were blocked with 1%
on-fat milk in Tris-buffered saline with 0.05% Tween20 detergent (Tween-20 TBS). The
blots were then incubated overnight at 4°C with the primary antibodies including
anti–adiponectin (Chemicon International, Inc.), anti–phospho-endothelial nitric oxide
synthase (eNOS) at Ser1177, anti–eNOS, anti–acetyl-lysine of Foxo1, anti–phospho-Thr24 of
Foxo1, anti–Foxo1, followed by incubation with a horseradish peroxidase–conjugated
secondary antibody. Anti–β-actin was used as internal immunoblot controls to confirm equal
protein loading. All of the other antibodies were obtained from Cell Signaling Technology
(Danvers, MA). Bands were visualized with an enhanced chemiluminescence detection
system (Pierce, Rockford, IL).

Chromatin Immunoprecipitation (ChIP). Chromatin was extracted from 3T3-L1 adipocytes
on day 9 after differentiation using the EZ-ChIP™ kit (Upstate, Billerica, MA, USA).
Adipocytes were treated with C3G for 24 h prior to crosslinking for 10 min with 1%
formaldehyde. Cells were then lysed and sonicated 3 times for 20 sec using a sonic
dismembrator (Fisher Scientific). Lysates were precleared with protein A agarose beads
(Millipore). FKHR or PPARγ antibody (Santa Cruz Biotechnology Inc.) 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). 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 years 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, recently diagnosed with diabetes mellitus, or 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 Labordiagnostik GmbH). 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 retro-orbital punctur. 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 were fractionated at 0.5 ml/min using a PBS containing 5 mM DTT as elution buffer
\((n = 4 \text{ per group})\). 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 \(\mu\text{g/ml}\). The
intra-assay and inter-assay 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 glycercyltrinitrate-
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 the mean ± SD. Comparison between
groups was analyzed using one-way analysis of variance followed by the Student-Newman-
Keuls test. \(P\) values <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 acetylcholine (Ach) in mice by using isometric tension studies. Compared to
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 as compared to 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 increased 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 approximately 2.5 fold higher in C3G group than in control group, and MMW oligomers were 1.7-fold higher C3G group than in control group, LMW oligomers were 1.5-fold higher C3G group than in 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 to 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 approximately 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 increased in adiponectin promoter activity (Fig.
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 media, 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 are relevant to humans and to rodent models, we treated rat adipocytes and primary human adipocytes with C3G for 24 h. In accordance with observations in 3T3L1 adipocytes results above, we observed significantly greater adiponectin mRNA expression (Fig. 3, A, B) and secretion (Fig. 3, C, D) in C3G-treated cells versus 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 physiologic 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 GW9662 exerted little influence on adiponectin expression and secretion (Fig. 4, A, B). However, pharmacological inhibition of Foxo1 with AS1708727 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-Δ256 (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-Δ256 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 Ser 256 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 treatment resulted in a 3.5-fold enhancement of C/EBPα binding to the 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 improve the 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 2 groups are shown in Tab. 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 2 groups. The serum adiponectin levels at 12 wk following anthocyanin administration were significantly increased in the anthocyanin group compared to baseline (Fig. 8A). Furthermore, there were significant increases in the FMD after the 12 wk anthocyanin supplementation (Tab. 1). There were no significant changes in serum adiponectin concentrations or FMD throughout the study in the placebo group. The changes in serum 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). No significant mean differences in the brachial diameter, baseline and hyperemic blood flow or GTND were observed between the 2 groups after the 12-wk intervention (Tab.1). 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 anthocyanin serum but not placebo serum (Fig. 9A). Anthocyanin serum also restored the reduced cGMP production under hyperglycemic conditions (Fig. 9B). Notably, depletion of adiponectin from the serum using lysine-Sepharose reduced anthocyanin serum-stimulated eNOS activation (Fig. 9C) and cGMP production (Fig. 9D). In addition, compared with quiescent HAECs, high glucose (HG) stimulated-HAECs showed a reduction in the adenylate 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 SQ22536 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 demonstrate 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 
hyperglycemic conditions. Thus, our current study indicate that adipose tissue can be an 
important target of anthocyanin through which releasing vasoactive hormone, 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 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 db/db induced by C3G. Correct 
oligomerization of adiponectin protein was essential for biological activity. 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 have been shown to possess a high level 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 μM, which is a commonly used dose in the in vitro study. At this dose, C3G produced a nearly 4-fold increase in adiponectin protein secretion into cultured media versus 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 PPARγ (18,29). We demonstrate that anthocyanin causes decreased acetylation of Foxo1, without changing its level of phosphorylation. Previous study reported that Foxo1 interaction with C/EBPα 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/EBPα 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 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 extended these findings and uncovered the molecular mechanism through which C3G regulated adiponectin transcription. More important, we elucidated that adipose tissue-derived adiponectin played
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 polyphenols in tertiary
prevention is relatively unknown (14). Our previous 3-month randomized, controlled trial
showed that anthocyanin intake improved endothelium-dependent vasodilation in
hypercholesterolemic individuals, effects that were thought to be mediated via NO-cGMP
pathway (47). We further explored whether pure anthocyanins would have a 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 wks,
we observed that anthocyanin supplementation dramatically improved endothelial function, as
determined by FMD measurement, while 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 cyclic AMP/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 high glucose. Further experiments demonstrated that adiponectin increased the
cyclic AMP 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.

**Abbreviations**

Ach, acetylcholine; C3G, cyanidin-3-O-β-glucoside; cGMP, cyclic guanosine monophosphate; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; FMD, flow-mediated dilatation; Foxo1-ADA, constitutively nuclear form of Foxo1; Foxo1-Δ256mutant, a dominant negative form of Foxo1; GTND, glyceraltrinitrate-induced dilatation; HAECs, human coronary artery endothelial cells; HG-DMEM, High-glucose DMEM; IMBX, 3-isobutyl 1-methylxanthine; Foxo1, forkhead box protein O1; HG, High glucose; LG-DMEM, low-glucose DMEM; NG, normal glucose; PKA, protein kinase A; Sirt1, silent mating type information regulation 2 homolog 1; SNP, sodium nitroprusside.

**ACKNOWLEDGMENTS**

This study was supported by grants from the National Natural Science Foundation of China (No. 81072301), the National Natural Science Foundation from Guangdong Province (No.S2012020011104). Project Supported by Guangdong Province Universities and Colleges Funded Scheme (2011). Guangzhou city science and technology project (12C22061588).

**DISCLOSURES**

All authors have no competing interests.

**AUTHOR CONTRIBUTIONS**

Yan Liu and Dan Li conducted the research, performed the statistical analysis and wrote the manuscript; Yan Liu, Dan Li, Yuhua Zhang and Ruifang Sun participated in the data collection and checked the data; Min Xia participated in the design of this study and wrote the manuscript. All authors have read and approved the final manuscript.

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positively influences serum triglyceride level in patients suffering from coronary

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Figure Legends

Fig. 1. Adiponectin mediates C3G-induced improvement of vascular reactivity in db/db mice. (A) Anti-adiponectin antibody (anti-Apn) abolished the effect of C3G to improve the 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) The phosphorylation level of eNOS Ser 1177 in aorta from db/db mice was determined by immunoblotting and expressed as fold of control. (D) Adiponectin secretion in the culture medium of subcutaneous, visceral, and perivascular adipose tissue isolated from db/db mice of Control and C3G group. Results are means ± SD of four experiments. *P<0.05 vs Control. (E) Adiponectin secretion in the culture medium of subcutaneous adipose tissue isolated from db/db mice of Control, C3G and C3G+anti-Apn group. Results are means ± SD of four experiments. *P<0.05 vs Control. (F) Serum adiponectin level was measured by an ELISA-based method. Results are means ± SD of three independent experiments. *P<0.05 vs Control or C3G+anti-Apn. n = 8 per group. (G) Representative blot (top) showing the distribution of adiponectin oligomers (HMW, MMW, and LMW) in db/db mice from Control and C3G group. Quantification of western blot data (bottom). Results are means ± SD. n = 4 per group. *P<0.05 vs corresponding Control. *P<0.05 vs corresponding Control.

Fig. 2. Induction of adiponectin expression and secretion by C3G in adipocytes. (A) Time-dependent induction of adiponectin transcription. Cultured adipocytes were incubated with C3G (25 μM) for the indicated times and adiponectin mRNA expression was quantified by qRT-PCR. The abundance of adiponectin mRNA, normalized to GAPDH, from C3G-treated cells were expressed as fold changes of value at 0 h, which was defined as 1. *P<0.05 vs. 0 h. (B) Dose-dependent induction of adiponectin transcription. 3T3-L1 adipocytes were treated with C3G for 24 h at the indicated concentrations and adiponectin mRNA expression by qRT-PCR. *P<0.05 or **P<0.01 vs. Control. (C) Adiponectin promoter-driven luciferase activity in the presence or absence of various concentrations of C3G. *P<0.05 or **P<0.01 vs. Control. Each bar represents the mean ± SD of duplicate determinations from four independent experiments. (D) 3T3-L1 adipocytes were treated with actinomycin D (5 μg/ml) in the presence or absence of C3G (25 μM) for different time points,
and adiponectin mRNA levels were determined by qRT-PCR. The normalized adiponectin mRNA signals were plotted as the percentage of the adiponectin mRNA remaining. Decay curves were plotted versus time. (E) 3T3-L1 adipocytes were treated with various concentrations of C3G (12.5, 25, 50 μM) for 24 h. Culture medium was immunoprecipitated for adiponectin using Catch-and-Release immunoprecipitation columns and kits. Immunoprecipitates containing equivalent amounts of total protein were subjected to immunoblotting using antibodies against adiponectin and β-actin. The blots shown are representative of three independent experiments (top) and quantification were expressed as fold of control which was defined as 1 (bottom). *P<0.05 vs. Control.

Fig. 3. C3G stimulate adiponectin expression and secretion in rat and human adipocytes. (A and C) Primary rat adipocytes and (B and D) primary human adipocytes from fresh human adipose tissues were treated with C3G (12.5 and 50 μM) for 24 h. Then, total RNA was isolated from the adipocytes lysates and subjected to qRT-PCR to measure the adiponectin mRNA expression levels in (A) rat and (B) human adipocytes. (C and D) Equal amounts of immunoprecipitated cell culture supernatants were subjected to western blot analysis to determine the adiponectin level from (C) rat and (D) human adipocytes using anti-β-actin as loading control. Representative blot from three independent experiments are shown (top) and quantification were expressed as fold of control which was defined as 1 (bottom). *P<0.05 vs. Control.

Fig. 4. PPARγ does not mediate C3G-induced adiponectin mRNA expression and secretion. 3T3-L1 adipocytes were incubated with C3G (50 μM) for 24 h in the absence or presence of the PPARγ antagonist GW9662 (10 μM). (A) Adiponectin mRNA expression and (B) secretion were determined by qRT-PCR and immunoblotting as indicated, respectively.

Fig. 5. Foxo1 mediates the C3G-induced upregulation of adiponectin. (A and B) 3T3-L1 adipocytes were incubated with C3G (50 μM) in the absence or presence of the Foxo1 inhibitor AS1708727 (20 μM) for 24 h. (A) Adiponectin mRNA expression was quantified by qRT-PCR analysis. Bars represent the mean ± SD of four independent experiments. (B)
Adiponectin secretion was determined by Western blot analysis. The blots shown are representative of four independent experiments. *$P<0.05$. (C and D) 3T3-L1 adipocytes were transfected with Foxo1 siRNA or Control siRNA and then treated with C3G (50 μM) for 24 h. (C) Adiponectin mRNA was measured by qRT-PCR, and results are expressed as fold of control. *$P<0.05$. Insert: The knockdown efficiency of Foxo1 siRNA on Foxo1 protein expression. (D) Adiponectin secretion was analyzed by Western blot. Representative data and quantitative analysis from three independent experiments are shown. (E) Representative western blot showing the relative levels of endogenous and ectopic Foxo1. Foxo1 Thr 24 phosphorylation, total Foxo1 in adipocytes infected with Foxo1-ADA and Foxo1-$\Delta$256 expression vectors. The $\Delta$256 form of Foxo1 lacks the epitope recognized by the Foxo1 antibody. (F) 3T3-L1 adipocytes transfected with Foxo1-ADA or Foxo1-$\Delta$256 were treated with C3G (50 μM) for 24 h. Adiponectin mRNA level was then assayed by qRT-PCR and results from four independent experiments are shown. *$P<0.05$ or #$P<0.05$.

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. (B) Foxo1 acetylation level and (C) Foxo1 transcriptional activity were measured as indicated, respectively. Data are expressed as mean ± SD from three 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 the three 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, then incubated with antibodies against Foxo1 and immunoblotted for Foxo1 and C/EBPα. Data are means ± SD from the three independent experiments. *$P<0.05$, **$P<0.01$ vs. Control.

Fig. 7. Sirt1 mediates C3G-induced Foxo1 deacetylation. (A-C) 3T3-L1 adipocytes transfected with either control or Sirt1 siRNA were treated with vehicle or C3G (50 μM) for
26 h. (A) The acetyl-lysine level was determined in Foxo1 immunoprecipitates (IP). Right: The knockdown efficiency of Sirt1 siRNA on Sirt1 protein expression. (B) Foxo1 transcriptional activity and (C) adiponectin mRNA level was measured, respectively and data represented results of three independent experiments. *P<0.05. (D) 3T3-L1 adipocytes were treated with various concentrations of C3G for 24 h. Then, Sirt1 deacetylase activity was determined by using a fluorometric kit. Data represent mean ± SD. *P<0.05 vs. Control.

Fig. 8. Anthocyanin induces adiponectin secretion and improves endothelial dysfunction in diabetic patients. (A) Serum adiponectin concentrations in placebo and anthocyanin group at baseline and after 12 wks intervention. *P<0.05 or **P<0.01. (B) Correlation between changes in adiponectin concentrations and FMD in anthocyanin group. (C) Correlation between changes in adiponectin concentrations and FMD in placebo group.

Fig. 9. Adiponectin activates cAMP-PKA-dependent eNOS activation in HG-incubated HAECs. (A) eNOS activity and (B) cGMP levels in high glucose (HG)-stimulated HAECs in the absence or presence of serum derived from placebo or anthocyanin group. (C) eNOS activity and (D) cGMP levels in HG-incubated HAECs cultured in anthocyanin serum or anthocyanin serum depleted of adiponectin. In some cultures, adiponectin-deficient serum was supplemented with 5 μg/ml adiponectin. Data represent mean ± SD, n = 16. *P<0.05.

Fig. 10. Adiponectin Increases NO Bioavailability through a cAMP-PKA signaling pathway. (A) Adenylate cyclase activity, (B) cAMP levels and (C) PKA in high glucose (HG)-stimulated HAECs in the absence or presence of serum derived from placebo or anthocyanin group. (D-E) HG-stimulated HAECs were incubated with serum from placebo or anthocyanin group in the presence of H89 or SQ22536. (D) eNOS phosphorylation levels and (E) cellular cGMP production were measured, respectively. *P<0.05.
Tab 1. The characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Placebo (n = 29)</th>
<th>Anthocyanin (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>62.3 ± 5.8</td>
<td>63.2 ± 7.3</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>17/12</td>
<td>17/12</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.7 ± 3.9</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>125 ± 6</td>
<td>131 ± 3</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>78 ± 8</td>
<td>75 ± 7</td>
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<tr>
<td>Fasting plasma glucose, mM</td>
<td>7.8 ± 1.5</td>
<td>7.3 ± 2.3</td>
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<tr>
<td>HbA1c, %</td>
<td>7.1 ± 0.8</td>
<td>6.9 ± 0.9</td>
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<tr>
<td>Total cholesterol, mM</td>
<td>4.9 ± 0.7</td>
<td>4.7 ± 0.8</td>
</tr>
<tr>
<td>LDL cholesterol, mM</td>
<td>3.12 ± 0.25</td>
<td>3.14 ± 0.37</td>
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<tr>
<td>HDL cholesterol, mM</td>
<td>0.95 ± 0.07</td>
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<tr>
<td>Triglycerides, mM</td>
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<td>2.13 ± 0.26</td>
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<td>Lipid medications, n</td>
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Tab 2. The metabolic data and endothelial function of the study groups

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<tr>
<td>Systolic blood pressure, mm Hg</td>
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<tr>
<td>Diastolic blood pressure, mm Hg</td>
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<tr>
<td>Fasting plasma glucose, mM</td>
<td>7.7 ± 1.5</td>
<td>7.5 ± 1.9</td>
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<tr>
<td>HbA1c, %</td>
<td>7.1 ± 0.8</td>
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<tr>
<td>Serum insulin, pM</td>
<td>52 ± 23</td>
<td>54 ± 31</td>
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<td>Serum C-peptide, nM</td>
<td>0.86 ± 0.29</td>
<td>0.84 ± 0.35</td>
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<td>Serum FFA, mM</td>
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<td>Brachial diameter, mm</td>
<td>4.02 ± 0.67</td>
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<td>Hyperemic blood flow, ml/min</td>
<td>352.4 ± 118.5</td>
<td>364.8 ± 128.4</td>
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<td>FMD, %</td>
<td>7.84 ± 2.03</td>
<td>8.02 ± 1.97</td>
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<tr>
<td>GTND, %</td>
<td>17.6 ± 4.9</td>
<td>17.4 ± 6.2</td>
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a No significant differences were found for any variable between the 2 groups at baseline via the unpaired Student t-test.

b Mean ± SD.

*P<0.05, **P<0.01 vs baseline, assessed by paired Student t-tests.
**Adiponectin expression level (Fold of Control)**

- **Control**
- **C3G**

**Subcutaneous adipose tissue**

- **adiponectin**
- **β-actin**

**Serum adiponectin levels**

- **HMW** (250 kDa)
- **MMW** (150 kDa)
- **LMW** (60 kDa)

**Adiponectin expression level**

- **Control**
- **C3G**

* Statistical significance indicated.
Figure 2

A. Adiponectin mRNA level (fold of control) over time.

B. Adiponectin mRNA level at different C3G concentrations.

C. Relative increase in adiponectin mRNA level over time.

D. Time (min) vs. Adiponectin mRNA level (% of control) for Control and C3G.
Figure 3

**A**

<table>
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<tr>
<th>Adiponectin mRNA level (fold of control)</th>
<th>Control</th>
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**B**

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**C**

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**D**

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<td>Human adipocytes</td>
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Figure 4

A. Adiponectin mRNA levels

B. Adiponectin and β-actin protein levels

GW9662

Control

C3G
E

<table>
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<tr>
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<th>Vector</th>
<th>Foxo1-ADA</th>
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<tr>
<td>Phos-Thr 24 Foxo1</td>
<td>+</td>
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<td>-</td>
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<tr>
<td>Foxo1</td>
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<tr>
<td>β-actin</td>
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<td>+</td>
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F

![Bar graph showing adiponectin mRNA level](Image)
Figure 6

A  

Ac-Foxo1  
Phos-Foxo1 (Ser 256)  
Foxo1

Foxo1 activation level (fold of control)

B  

Ac-Foxo1  
Foxo1

Foxo1 activation level (fold of control)

C3G (μM)

0  12.5  25  50

*  **

C3G (h)

0  3  6  12  24

*  **
**E**

**C/EBPα**

**Foxo1**

**D**

**Ferro1 bound**

**Input**

**C**

**Foxo1 transcriptional activity (Fold of Control)**

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<td>1.0</td>
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**E**

**IP: Foxo1**

**C/EBPα**

**Foxo1**

**F**

**Relative enrichment (Fold of control)**

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Figure 7

A. Foxo1 translocation activity

B. Sirt1 deacetylase activity

C. Adiponectin mRNA level
Figure 8

A

Serum adiponectin levels (µg/mL)

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<tr>
<td>Anthocyanin</td>
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B

\[ r = 0.661, P < 0.001 \]

C

\[ r = 0.137, P = 0.469 \]

Anthocyanin group

Placebo group
Figure 10

A. Adenosyl cyclase enzyme activity
B. CAMP levels
C. PKA activity (AU)
D. Western Blot images
E. Cellular CAMP levels