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Biochemical and Biophysical Research Communications 316 (2004) 149–157
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Anthocyanin enhances adipocytokine secretion and adipocyte-specific gene expression in isolated rat adipocytes☆

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Received 3 February 2004

Abstract

Adipocyte dysfunction is strongly associated with the development of obesity and insulin resistance. It is accepted that the regulation of adipocytokine secretion or the adipocyte-specific gene expression is one of the most important targets for the prevention of obesity and amelioration of insulin sensitivity. In this study, we demonstrated that anthocyanins (cyanidin or cyanidin 3-glucoside) have the potency of a unique pharmacological function in isolated rat adipocytes. Treated adipocytes with anthocyanins enhanced adipocytokine (adiponectin and leptin) secretion and up-regulated the adipocyte specific gene expression without activation of PPARγ in isolated rat adipocytes. The gene expression of adiponectin was also up-regulated in white adipose tissue in mice fed an anthocyanin supplemented diet. As one of the possible mechanisms, AMP-activated protein kinase activation would be associated with these changes, nevertheless, the AMP:ATP ratio was significantly decreased by administration of the anthocyanins. These data suggest that anthocyanins have a potency of unique therapeutic advantage and also have important implications for preventing obesity and diabetes.

Keywords: Anthocyanin; Cyanidin; Adipocyte; Adipocytokine; Adiponectin; Adipocyte-specific gene; Peroxisome proliferator-activated receptor

Adipocyte is the primary site of energy storage and accumulates triacylglycerol during nutritional excess. In recent years, it is well known that adipocyte dysfunction plays an important role in the development of obesity and insulin resistance. Adipocyte synthesizes and secretes biologically active molecules called adipocytokines [1]. For example, leptin is the product of the ob gene and is secreted from adipocytes, and reduces food intake and increases energy expenditure [2]. Adiponectin is one of the most important adipocytokines, and is specifically and highly expressed in adipocytes. The plasma adiponectin concentration and mRNA expression level are decreased in the obese and insulin resistant state [3,4]. The administration of adiponectin improves insulin action accompanied by increases in fatty acid oxidation and a decreased triacylglycerol level in muscle [5,6].

There are some drugs which are the target for regulation of the adipocyte function to improve insulin sensitivity or glucose homeostasis. The peroxisome proliferator-activated receptor (PPAR)γ is the master regulator of adipocyte differentiation and controls various kinds of adipocyte specific gene expressions. Thiazolidinediones (TZD) are some of the synthetic PPAR ligands, and used for antidiabetic drugs through their

☆ *Abbreviations:* AMPK, AMP-activated protein kinase; aP2, adipocyte fatty acid binding protein; BSA, bovine serum albumin; C3G, cyanidin 3-*O*-β-D-glucoside; Cy, cyanidin; ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acid; HPLC, high-performance liquid chromatography; KRBH, Krebs–Ringer bicarbonate buffer; LPL, lipoprotein lipase; MAPK, mitogen-activated protein kinase; PCC, purple corn color; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; TTBS, Tris–HCl-buffered saline containing 0.05% Tween 20; TZD, thiazolidinediones; UCP, uncoupling protein.

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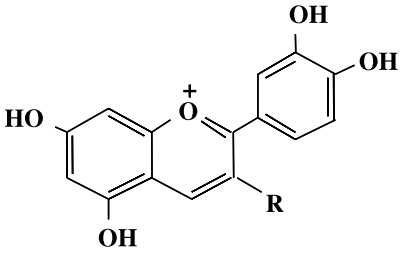
doi:10.1016/j.bbrc.2004.02.031

effects on the adipocyte differentiation and activation of adipocyte genes [7,8]. Fibrates are hypolipidemic drugs whose effects are mediated by the activation of PPARα. The administration of the drug also increases the fatty acid oxidation and the change may be involved in the reduction of fat deposits and improvement of insulin sensitivity [9]. Metformin ameliorates hyperglycemia without stimulating insulin secretion. The administration of the drug leads to the activation of AMP-activated protein kinase (AMPK) which has been implicated as a potential target of type 2 diabetes mellitus and obesity [10].

Recently, much attention has been focused on some food factors that may be beneficial for the prevention of body fat accumulation and possibly reduce the risk of diabetes and heart disease. Although some drugs are used for the therapy of obese-related metabolic diseases or possibility discussed as preventing body fat accumulation, there has been little evidence that food factors themselves are directly beneficial for the improvement of the dysfunction of adipocyte responsible for adipocytokine secretion and lipid metabolism.

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. They are widely distributed in the human diet through crops, beans, fruits, vegetables, and red wine [11], suggesting that we ingest significant amounts of anthocyanins from plant-based daily diets. In general, anthocyanin pigments are stable under acidic conditions, but are unstable and rapidly broken down under neutral conditions [12]. Therefore, anthocyanins have not been recognized as a physiological functional food factor [12]. However, we demonstrated that cyanidin 3-*O*-β-D-glucoside (C3G) (Fig. 1), which is a typical anthocyanin, had antioxidative and anti-inflammatory activities based on in vitro and in vivo studies [13–16]. These findings suggest that C3G has more beneficial effects beyond its antioxidant activity.

Moreover, we have previously demonstrated that dietary anthocyanin significantly suppressed the development of obesity, normalized hypertrophy of the adipocytes in the epididymal white adipose tissues, and ameliorated hyperglycemia induced by high-fat diet feeding of C57BL/6J mice [17]. These results suggest that anthocyanins can regulate obesity and insulin sensitivity associated with adipocytokine secretion and PPARγ activation in adipocytes. To clarify the molecular action and mechanism of anthocyanin responsible for the amelioration of insulin sensitivity and prevention of obesity through regulation of the adipocyte function, the present study was designed to show the administration of C3G or cyanidin (Cy) (Fig. 1), which is an aglycon of C3G that enhances adipocytokine secretion and up-regulates adipocyte specific genes without activation of PPARγ. As one of the possible mechanisms of the anthocyanin-mediated effects, we also examined the effect of anthocyanins on AMPK activation which plays a key role in the metabolic regulation of lipids and glucose uptake in adipocyte.



R = -*O*-β-D-glucose; cyanidin 3-glucoside (C3G)
R = OH; cyanidin (Cy)

Fig. 1. Chemical structure of cyanidin 3-*O*-β-D-glucoside (C3G) and cyanidin (Cy).

Materials and methods

Chemicals. Cy and C3G were obtained from Extrasynthèse (Genay, France) and their purities were greater than 99%. Collagenase (type II) was purchased from Sigma Diagnostics (St. Louis, MO). Bovine serum albumin (BSA) fraction V was obtained from Nakarai Tesque (Kyoto, Japan). T174 TZD, a specific agonist for PPARγ [18], was kindly provided by Tanabe Seiyaku (Osaka, Japan).

Isolation of adipocytes from rat epididymal adipose tissue. Rat adipocytes from epididymal adipose tissue were isolated using the method of Rodbell [19]. Briefly, male Wistar rats, 7 weeks of age (Japan SLC, Hamamatsu, Japan), were used and maintained at 23 ± 3 °C under an automatic lighting schedule (08:00 to 20:00 h). The rats were allowed free access to water and a laboratory nonpurified diet (CE-2, CLEA, Tokyo, Japan) for 3 days. This experimental design was approved by the Animal Experiment Committee, Nagoya University, and the rats were maintained in accordance with the guidelines. After 3 days of feeding the diets, the rats were killed by decapitation and the epididymal adipose tissues were removed under sterile conditions. The tissues were minced in 30 mM Hepes–Krebs–Ringer bicarbonate buffer (KRBH) containing 0.5% BSA and collagenase (76.6 U/ml). After incubation at 37 °C for 90 min, the digestion was filtered through a sterile 250 μm nylon mesh. The filtrate was washed for four times with 30 mM Hepes–KRBH containing 0.5% BSA. The washed adipocytes (100 μl packed volume per dish) were suspended in Dulbecco's modified Eagle's medium containing BSA (0.5%), penicillin (100 U/ml), and streptomycin (100 μg/ml). The adipocytes were treated with 100 μM Cy, C3G or vehicle (0.1% dimethyl sulfoxide) for 24 h at 37 °C in a humidified atmosphere (5% CO_2/95% air).

Isolation of total RNA and measurement of gene expression level. Total RNA from the adipocytes was isolated with Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's directions. Total RNA (2.0 μg) was incubated with 9 mM of the oligo(dT) primer at 65 °C for 15 min, then reverse transcribed to cDNA in a reaction mixture containing buffer, 1.3 mM dNTP (Gibco-BRL, Grand Island, NY), 10 mM dithiothreitol, 20 U Rnase inhibitor (Gibco-BRL, Grand Island, NY), and 200 U Moloney murine leukemia virus reverse transcriptase (MBI Fermentas, Lithuania) at 37 °C for 3 h, and then heated at 94 °C for 2 min to terminate the reaction. The reverse transcription-polymerase chain reaction (RT-PCR) was performed in a final 25 μl containing 0.5 μl of the RT first-strand cDNA product, 1 μM of each forward (F) and reverse (R) primer, 75 mM Tris–HCl (pH 8.8) containing 1 mg/L Tween 20, 0.2 mM dNTP, 2 mM $MgCl_2$, and 1.5 U *Taq* DNA polymerase (MBI Fermentas, Lithuania). Preliminary

experiments were carried out with various cycles to determine the nonsaturating conditions of the PCR amplification for all the genes studied. The primers were:

rat adiponectin (Gene Accession No: NM_144744),
(F): 5′-CTCCACCCAAGGAAACTTGT-3′,
(R): 5′-CTGGTCCACATTTTTTTCCT -3′ (502 bp);
mice adiponectin (Gene Accession No: NM_009605),
(F): 5′-CTTTGGTCCCTCCACCCAAG-3′,
(R): 5′-GAGAACGGCCTTGTCCTTCT-3′ (472 bp);
leptin (Gene Accession No: NM_008493),
(F): 5′-GTTTTGGAGCAGTTTGGATC-3′,
(R): 5′-GCATATGGGAAGTTTCACAA-3′ (522 bp);
PPARγ (Gene Accession No: NM_013124),
(F): 5′-CATTTCTGCTCCACACTATGAA-3′,
(R): 5′-CGGGAAGGACTTTATGTATGAG-3′ (552 bp);
lipoprotein lipase (LPL) (Gene Accession No: BC003305),
(F): 5′-GATCCATGGATGGACGGTAA-3′,
(R): 5′-TGGGATAAATGTCAACATGC-3′ (472 bp);
adipocyte fatty acid binding protein (aP2) (Gene Accession No: AE144756),
(F): 5′-GGGGACCTGGAAACTCGTCT-3′,
(R): 5′-CCCATCAAGTTATTGTAGAG-3′ (439 bp);
uncoupling protein 2 (UCP2) (Gene Accession No: AB005143),
(F): 5′-CCTGTATTGCAGATCTCATC-3′,
(R): 5′-GCTCAGTACAGTTGACAATG-3′ (512 bp), and
β-actin (Gene Accession No: NM_031144)
(F): 5′-CGTGGGCCGCCCTAGGCACCA-3′,
(R): 5′-CTCTTTGATGTCACGCACGATTTC-3′ (543 bp).

The PCR cycle numbers were 17 or 20 cycles for rat or mice adiponectin, 19 cycles for β-actin, 22 cycles for LPL and PPARγ, 27 cycles for aP2 and leptin, and 29 cycles for UCP2. Each cycle consisted of denaturation at 94 °C for 30 s, primer annealing at 50 °C for PPARγ, 56 °C for leptin, LPL, aP2, and UCP2, 58 °C for rat adiponectin and β-actin, and 60 °C for mice adiponectin, and primer extension at 72 °C for 30 s. A final 10 min primer extension step at 72 °C was performed on all of the samples. The products were run on 10 g/L agarose gels and stained with ethidium bromide. The relative density of the bands was evaluated using an ATTO Lane Analyzer 10H Software Densitograph (Atto, Tokyo, Japan). All the measured PCR products were normalized to the amount of cDNA of β-actin in each sample.

Measurement of adiponectin mRNA level in the mice epididymal white adipose tissue. Male 12 C57BL/6 mice, 4 weeks of age (Japan SLC, Hamamatsu, Japan), were used and maintained at 23 ± 3 °C under an automatic lighting schedule (08:00 to 20:00 h). The mice were randomly divided into two groups and assigned to the control or control diet supplemented with C3G-rich purple corn color (PCC) diet. PCC was added to the control diet at a C3G concentration of 2 g/kg diet. The diets were replaced once every 3 days to prevent deterioration of the PCC. This experimental design was approved by the Animal Experiment Committee, Nagoya University, and the mice were maintained in accordance with the guidelines. After 12 weeks of being fed the diets, the mice were killed by decapitation and the blood was removed. The epididymal white adipose tissues were removed according to the defined anatomical landmarks. Total RNA was isolated and the mRNA level was measured as previously described.

PPARγ reporter assay. Measurement of the PPARγ ligand activity was performed in a GAL4-PPAR-γ chimera assay system using the previously described method of Takahashi et al. [20]. Briefly, CV1 monkey kidney cells were obtained from the American Type Culture Collection. CV1 cells were grown in maintenance medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 mg/ml penicillin and streptomycin) at 37 °C in 5% CO_2. Two plasmids, pM-hPPAR-γ and p4xUASg-tk-luc, were transfected into cells cultured in 24-well plates using LipofectAMINE (Invitrogen, CA, USA). pM-hPPAR-γ is a plasmid expressing a fusion protein of the GAL4 DNA-binding domain and human PPARγ ligand-binding domain, and p4xUASg-tk-luc is a reporter plasmid containing four copies of a 17-mer upstream activating sequence (UAS) for the GAL4 DNA-binding domain and thymidine kinase gene promoter (tk-promoter) in front of luciferase cDNA. After transfection, the cells were cultured for 24 h, and Cy, T-174 TZD or vehicle for ligand assay was added to the medium at appropriate concentrations. After an additional 24-h incubation, the cells were lysed for luciferase assay using a Dual-Luciferase Reporter Gene Assay system (Promega, Tokyo, Japan) according to the manufacturer's protocol.

Measurement of adipocytokine levels. The adiponectin concentration in the media and mice serum, leptin concentration in the media were measured by an enzyme-linked immunosorbent assay (ELISA) using commercial assay kits according to the manufacturer's directions (mouse/rat adiponectin ELISA kit, Otsuka Pharmaceutical, Tokyo, Japan, rat leptin ELISA kit, Morinaga, Yokohama, Japan).

Immunoblot analysis of phospho-AMPK (Thr 172) protein. The cells treated with or without anthocyanins were washed in ice-cold phosphate-buffered saline and then lysed with digitonin buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaF, 30 mM glycerol phosphate, 0.25% sucrose, 1 mM metavanadate, 1.5 mM phenylmethylsulfonyl fluoride, and 0.4 mg/ml digitonin) on ice for 2 min [21]. The cell lysate was centrifuged at 10,000*g* for 10 min at 4 °C. The protein concentration of the obtained supernatant was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Aliquots of the supernatant were treated with Laemmli sample buffer for 5 min at 100 °C [22]. The samples (20 μg protein) were then loaded into the SDS–PAGE system. The resulting gel was transblotted on a nitrocellulose membrane (Hybond ECL, Amersham–Pharmacia Biotech, Tokyo, Japan). It was next blocked by 5% skim milk for 1 h at room temperature. After washing with 20 mM Tris–HCl-buffered saline containing 0.05% Tween 20 (TTBS), the membrane sheets were then reacted with Anti-phospho-AMPK antibody (Thr 172, 1:1000 dilutions) (Cell Signaling Technology, USA) for 16 h at 4 °C. After washing with TTBS, it was reacted with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2000 dilutions) for 1 h at room temperature. After washing, the immunoreactivity was visualized using the ECL reagent and the relative density was evaluated with a ATTO Lane Analyzer 10H Densitograph Software (Atto, Tokyo, Japan).

ATP, ADP, and AMP concentrations in adipocytes. The intracellular ATP, ADP, and AMP concentrations were measured using high-performance liquid chromatography (HPLC). Briefly, the cells were washed with in ice-cold phosphate-buffered saline and then extracted with 100 μl of 5.5% $HClO_4$. The extract was centrifuged at 10,000*g* for 10 min at 4 °C. Aliquots of the supernatant were used for the HPLC analysis. HPLC was carried out on a Develosil RPAQUOUS column (Nomura Chemical, Aichi, Japan, 4.6 mm × 150 mm) with a photodiode array detector (MD-910, Jasco, Tokyo, Japan), and 1% methanol in 100 mM KH_2PO_4 (pH 6.0) as the solvent at a flow rate of 1.0 ml/min.

Statistical analysis. The data were expressed as means ± SEM. The differences among the means were analyzed by Fisher's protected least-significant difference test following one-way ANOVA or unpaired Student's *t* test. Differences with *P* values <0.05 were considered significant. All the statistical analyses were performed using the StatView version 5.0 software for Macintosh (SAS Institute, Cary, NC, USA).

Results

Adipocytokine secretion and mRNA level after treatment of anthocyanins

Fig. 2 shows the effects of the anthocyanins on leptin and adiponectin secretion into the medium and

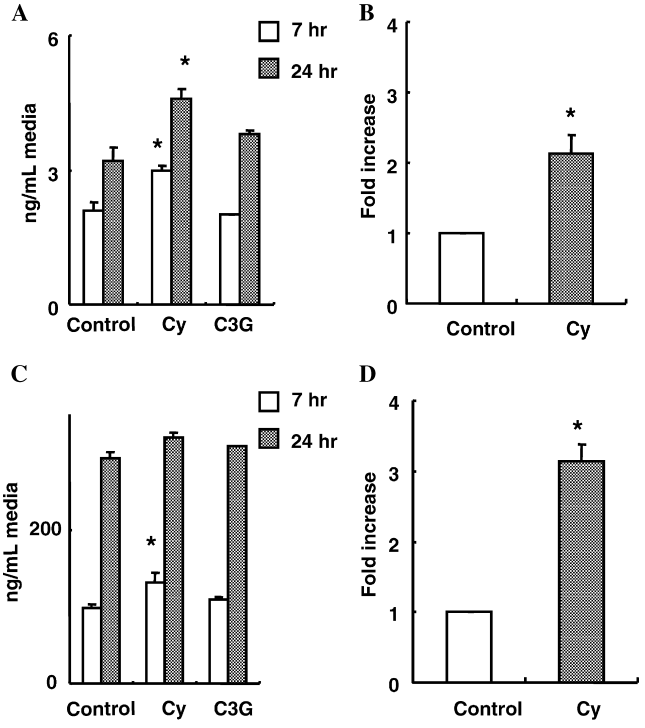


Fig. 2. Effects of anthocyanins on leptin and adiponectin expression in rat adipocytes. (A) Leptin secretion in media, (B) gene expression level of leptin, (C) adiponectin secretion in media, and (D) gene expression level of adiponectin. The gene expression level was expressed relative to the control group (= 1.0) after normalization using the β-actin gene expression level. Values are means ± SEM, $n = 3$. *Significantly different at $P < 0.05$ compared to the control in each period.

mRNA level. Treatment of the adipocytes with Cy significantly enhanced the leptin secretion into the media throughout the experimental period (7 h, 1.4-fold; 24 h, 1.4-fold) (Fig. 2A). However, treatment with C3G did not enhance its secretion compared to the control. The leptin mRNA level was also significantly increased in the Cy treated group (24 h, 2.1-fold) (Fig. 2B). Adiponectin secretion into the media was significantly enhanced in the Cy treated group in 7 h (1.3-fold) (Fig. 2C). The adiponectin mRNA level was significantly increased in the Cy treated group (24 h, 3.1-fold), although the secretion was not significantly elevated compared to the control group in 24 h (Fig. 2D).

The effects of anthocyanin on mRNA level and adiponectin secretion in mice

To further elucidate the effect of anthocyanin on the adiponectin secretion and mRNA expression in vivo, mice were fed the control or control + anthocyanin (C3G-rich PCC) diet for 12 weeks. The adiponectin mRNA level in epididymal white adipose tissue was significantly increased in the anthocyanin group compared to the control group (1.7-fold) (Fig. 3A).

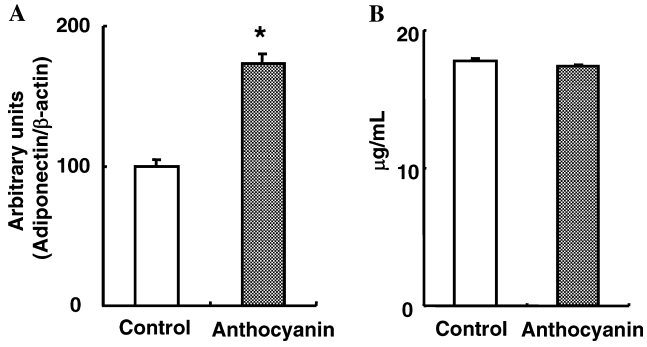


Fig. 3. Gene expression level in epididymal white adipose tissue (A) and serum concentration of adiponectin (B) in mice fed the control or anthocyanin (C3G)-rich purple corn color (PCC) diet. The gene expression level was expressed by assigning 100 as the value in the control group after normalization using the β-actin gene expression level. Values are means ± SEM from six mice. *Significantly different at $P < 0.05$ compared to the control.

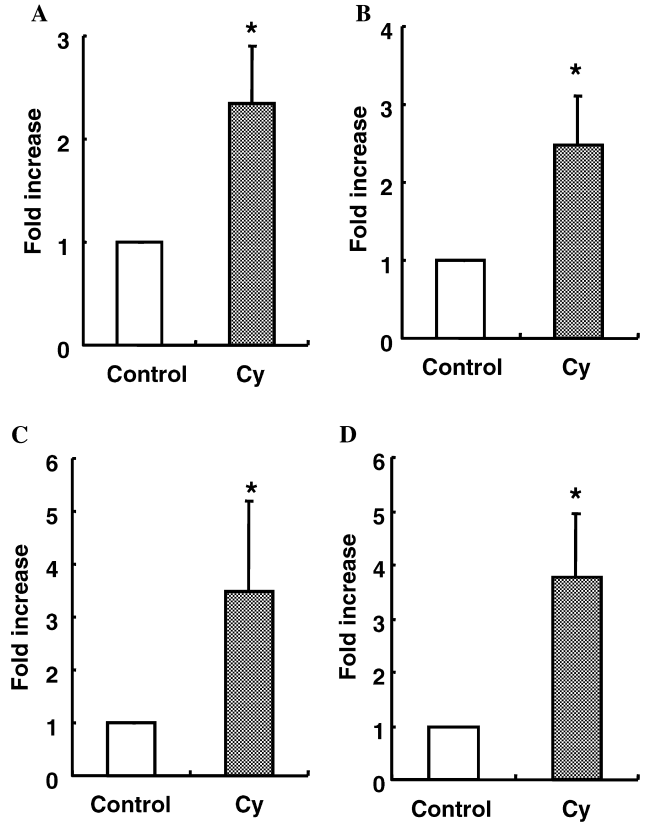


Fig. 4. Gene expression level of peroxisome proliferator-activated receptor (PPAR)γ target adipocyte specific genes and PPARγ in adipocytes treated with Cy for 24 h. (A) Lipoprotein lipase (LPL), (B) adipocyte fatty acid binding protein (aP2), (C) uncoupling protein 2 (UCP)2, and (D) PPARγ. The gene expression level was expressed relative to the control group (= 1.0) after normalization using the β-actin gene expression level. Values are means ± SEM from three independent experiments. *Significantly different at $P < 0.05$ compared to the control.

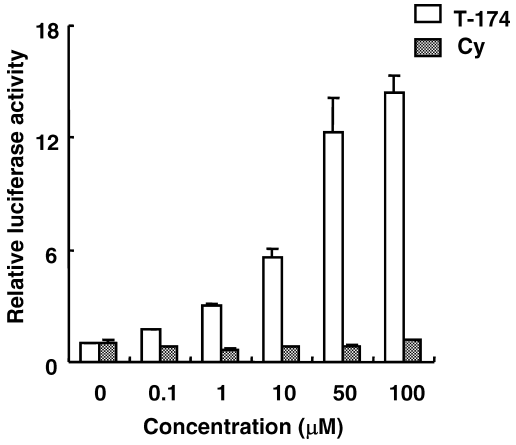


Fig. 5. PPARγ transcriptional activity of cyanidin (Cy) or T-174. The activity of a vehicle control (0 μM) was set at 1.0 and the relative luciferase activities were presented as fold induction to that of the vehicle control. Values are means ± SEM, $n = 4$.

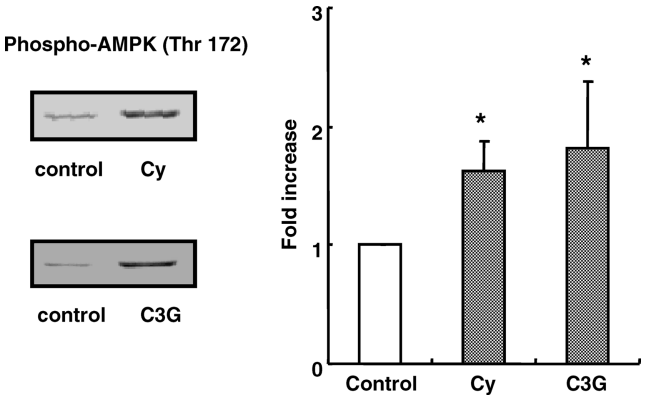


Fig. 6. The immunoblot analysis of the phospho-AMP-activated protein kinase (AMPK) protein (Thr 172) in adipocytes treated with anthocyanins for 24 h. Values are means ± SEM from two or three independent experiments. *Significantly different at $P < 0.05$ compared to the control.

The serum adiponectin concentration did not differ between the control and anthocyanin groups (Fig. 3B).

Effects of Cy on PPARγ target adipocyte specific gene expression

The mRNA level of the PPARγ targeted gene (LPL, aP2, and UCP2) and PPARγ itself in adipocytes treated with Cy for 24 h are shown in Fig. 4. These mRNA levels were significantly increased by the administration of Cy (LPL, 2.4-fold; aP2, 2.5-fold; UCP2, 3.5-fold; and PPARγ, 3.8-fold) compared to the control.

PPARγ ligand activity of Cy

Fig. 5 shows the PPARγ ligand activity of Cy using a GAL4-PPAR-γ chimera assay system. Positive control of T-174, a specific agonist for PPARγ [18], stimulated the PPARγ-dependent luciferase activities in a dose-dependent manner. PPARγ activation by 100 μM T-174 was approximately 15-fold as compared with the vehicle control. However, Cy did not induce luciferase activity even if its concentration was increased to 100 μM.

AMPK activation in adipocytes treated with anthocyanins

Recent reports have shown that leptin or adiponectin enhances AMPK phosphorylation which resulted in fatty acid oxidation [23,24]. Based on these reports and our findings, we examined the AMPK activation in adipocytes treated with anthocyanins. Fig. 6 shows the detection of phosphorylated AMPK (Thr-172) using a phosphospecific antibody in adipocytes treated with Cy or C3G for 24 h. Significant elevation of the phospho-AMPK (Thr-172) protein level was observed in both the Cy and C3G treated groups compared to the control groups.

ATP, ADP, and AMP concentrations in adipocytes

It is understood that AMPK functions as a fuel gauge to monitor the cellular energy status, and it is also known that AMPK is activated by AMP allosterically and by phosphorylation mediated by AMPK-kinase [25]. An increase in the AMP:ATP ratio activates

Table 1
AMP:ATP ratio and ATP, ADP, and AMP concentration in rat adipocytes treated with anthocyanins

	AMP:ATP ratio	ATP	ADP (nmol/dish)	AMP
7 h				
Control	0.416 ± 0.021^{a}	1.054 ± 0.086^{b}	0.440 ± 0.048^{a}	0.438 ± 0.041^{a}
Cy	0.119 ± 0.018^{b}	1.685 ± 0.060^{a}	0.410 ± 0.038^{a}	0.202 ± 0.034^{b}
C3G	0.105 ± 0.014^{b}	1.696 ± 0.240^{a}	0.446 ± 0.103^{a}	0.183 ± 0.047^{b}
24 h				
Control	0.335 ± 0.036^{a}	0.830 ± 0.037^{a}	0.437 ± 0.065^{a}	0.281 ± 0.041^{a}
Cy	0.238 ± 0.014^{b}	0.696 ± 0.041^{b}	0.327 ± 0.029^{a}	0.165 ± 0.003^{b}
C3G	0.195 ± 0.012^{b}	0.724 ± 0.012^{a}	0.327 ± 0.029^{a}	0.141 ± 0.008^{b}

Data were obtained from three experiments and represent means ± SE. The values with different superscript letters are significantly different ($P < 0.05$).

AMPK, although AMPK can also be stimulated through the AMP:ATP ratio-independent pathway [26]. Therefore, we examined whether or not phosphorylation of AMPK in adipocytes treated with anthocyanins is due to an increase in the AMP:ATP ratio. Table 1 shows the AMP:ATP ratio and ATP, ADP, and AMP concentrations in adipocytes treated with Cy or C3G. The AMP:ATP ratio significantly decreased in both of the anthocyanin treated groups compared to the control group throughout the experimental period (Cy, decreased by 71.5% in 7 h and 39.0% in 24 h; C3G, decreased by 74.8% in 7 h and 41.8% in 24 h). The AMP concentrations were also significantly decreased in both of anthocyanin treated groups compared to the control group. On the contrary, the ATP concentrations were significantly increased in both of the anthocyanin treated groups within 7 h compared to the control group.

Discussion

Adipocyte dysfunction plays an important role in the development of obesity and insulin resistance. Some drugs are used for the therapy of obese-related metabolic diseases or in the discussion on the possibility of preventing body fat accumulation. However, there has been little evidence that food factors themselves directly modulate the adipocyte function including adipocytokine secretion or adipocyte specific gene expression. In this study, we demonstrated that anthocyanins have the potency of a unique pharmacological function in adipocytes.

The regulatory mechanism for the gene expression of adiponectin and its secretion from adipocytes is complex. Maeda et al. [27] and Combs et al. [28] showed that TZD, one of the synthetic PPAR ligands and used for antidiabetic drugs, increased the expression and secretion of adiponectin in mice and humans. Maeda et al. [27] also demonstrated that TZD enhanced the adiponectin promoter activity in 3T3-L1 adipocytes. However, Gustafson et al. [29] showed that the activation of adiponectin and aP2 genes can be differentially regulated in adipocytes. Fasshauer et al. [30,31] reported that treatment with insulin, tumor necrosis factor α, interleukin-6 or dexamethasone suppressed the adiponectin gene expression, and pretreatment with p44/42 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and p70S6kinase inhibitors partially reversed the inhibitory effect in 3T3-L1 cells. The novel findings of this study are that the treatment of adipocytes with Cy enhanced adiponectin and leptin secretion and their mRNA levels, and also dietary C3G significantly increased adiponectin mRNA level in mice. This is the first report that anthocyanins, one of the food factors and not a drug, modulate these expression levels. However, the present study also proposed some questions as to why the structure of anthocyanins (glucoside or aglycon) differently affected the gene expression and secretion of these adipocytokines, and the elevation of the adiponectin gene expression by anthocyanins was not reflected on that of the serum protein level in vivo. The exact structure–activity relationship and secretory mechanism should be investigated. An increase in the gene expression of adiponectin will be beneficial for insulin sensitizing and the control of obesity as well as the atherogenic and anti-inflammatory processes in endothelial cells. Anthocyanins can provide a unique therapeutic advantage involved in the regulation of the adipocyte function to improve insulin sensitivity.

Yamauchi et al. [32] succeeded in the cloning of cDNAs encoding two adiponectin receptors (AdipoR1 and AdipoR2). These receptors have not be found in adipocytes. However, Wu et al. [33] demonstrated that the treatment of rat adipocytes with the globular domain of adiponectin increased in the glucose uptake and AMPK activation without stimulating the tyrosine phosphorylation of the insulin receptor or insulin receptor substrate-1 and Akt. Adiponectin may modulate the adipocyte function as well as leptin by the autocrine system. Minokoshi et al. [25] demonstrated that leptin activates AMPK, and the activation is strongly associated with the enhancement of fatty acid oxidation and suppression of triacylglycerol accumulation. This activation is also performed in adipocytes to prevent excess lipid accumulation in them [34]. It is noteworthy and the second novel finding of the present study that the treatment of adipocytes with anthocyanins markedly increased the phosphorylation of AMPK (Thr-172). Based on Wu et al. and Minokoshi et al. reports, the AMPK activation by anthocyanins may be linked to adiponectin and/or leptin secretion and gene expression. There are two possibilities that anthocyanins can directly activate AMPK, or increase the gene expression and protein secretion of adiponectin and leptin by anthocyanins inducing AMPK activation. The target molecule for anthocyanins should be identified.

In this study, the PPARγ target adipocyte specific genes (LPL, aP2, and UCP2) and PPARγ itself were up-regulated by the treatment of Cy. Surprisingly, Cy did not stimulate the PPRE-dependent luciferase activities, indicating that up-regulation of the adipocyte specific genes by Cy is not due to stimulation of the PPARγ ligand activity, but is due to a PPARγ-independent mechanism. Recent reports showed that the administration of TZD or inflammatory cytokines activated p38 MAPK and resulted in the up-regulation of UCPs and fatty acid oxidation through the activation of the PPARγ coactivator-1 [35–37]. The administration of the reduced form of glutathione with TZD inhibited the up-regulation of UCP1 accompanied with

p38 MAPK activation in brown adipocyte [37]. Lennon et al. [38] reported that TZD and 15-deoxy-$^{\Delta 12-14}$-prostaglandin J_2, which is recognized as a PPARγ ligand, activates the MAPK signaling pathways through PPARγ-independent mechanisms involving reactive oxygen species (ROS). Hou et al. [39] showed that anthocyanins may trigger an apoptotic death program in HL-60 cells through oxidative stress involved in the MAPK signaling pathway. Based on these reports, there may be a possible pathway that Cy functions as a weak pro-oxidant rather than as an antioxidant, and ROS generated from Cy induced activation of p38 MAPK and the PPARγ target genes that were up-regulated. Yamauchi et al. [32] showed that the treatment of myocyte with adiponectin induced p38 MAPK activation accompanied with AMPK activation. Also, recent reports showed that the UCPs gene expression was increased by the activation of AMPK in β-cells or rat skeletal muscle treated with aminoimidazole-4-carboxamide-1-β-D-ribofuranoside [40–42]. The interaction of AMPK, ROS, and p38 MAPK activation with the adipocyte specific gene expression should be investigated.

There is another question about how AMPK is activated by anthocyanins. It is well known that AMPK is activated by an increase in the AMP:ATP ratio that involves reduction of ATP or elevation of the AMP concentration in cells. Minokoshi et al. showed that leptin stimulates AMPK after 6 h without the AMP:ATP ratio in soleus muscle [25]. It is noteworthy that the AMP:ATP ratio decreased by the treatment adipocytes with anthocyanins within 24 h. TZD and metformin also stimulates AMPK without a change in the AMP:ATP ratio, but its ratio is not significantly decreased by the administration of these drugs [10,43]. Anthocyanins activate AMPK regardless of the decrease in the AMP:ATP ratio, indicating that its mechanism for the activation by anthocyanins is independent of its ratio and may also be different from that of TZD and metformin.

In conclusion, we have clearly demonstrated for the first time that anthocyanins enhance adipocytokine (adiponectin and leptin) secretion, the expression of PPARγ and adipocyte specific genes (LPL, aP2, and UCP2) in isolated rat adipocytes. However, the up-regulation of the adipocyte specific genes was not due to the stimulation of the PPARγ ligand activity. There may be multiple mechanisms for the regulation of these adipocytokine gene expressions and their secretion by anthocyanins. As one of the possible mechanisms, AMPK activation would be associated with these changes, nevertheless the AMP:ATP ratio was significantly decreased by the administration of the anthocyanins. Anthocyanins, which are one of the most popular plant pigments as food factors, have the potency of a unique therapeutic advantage responsible for the regulation of the adipocyte function to improve insulin sensitivity and the target molecule may be different than that of TZD. Our findings provide a biochemical basis for the use of anthocyanins, which can also have important implications for preventing obesity and diabetes.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research (No. 15500565) from the Japanese Ministry of Education, Culture, Sports, Science and Technology and PROBRAIN Project from Bio-oriented Technology Research Advancement Institution, Japan. We thank Dr. Ryuichi Moriyama (Nagoya University) and Dr. Chie Morimoto (Ehime University) for their useful discussions and technical assistances about isolation of rat adipocytes.

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