**Increasing cAMP levels of preadipocytes by cyanidin-3-glucoside treatment
induces the formation of beige phenotypes in 3T3-L1 adipocytes**

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Keywords: Cyanidin-3-glucoside, 3T3-L1 adipocytes, mitochondria, beige adipocyte, differentiation

Abstract

Obesity is a serious health problem and a major risk factor for the onset of several diseases such as heart disease, diabetes, stroke, and cancer. The conversion of white adipocytes to brown-like adipocytes, also called beige or **brite** adipocytes, by pharmacological and dietary compounds has gained attention as an effective treatment for obesity. Cyanidin-3-glucoside (Cy3G), a polyphenolic compound contained in black soybean, blueberry and grape, has several anti-obesity effects. However, there are no reports on the role of Cy3G in the induction of differentiation of preadipocytes to beige adipocytes and corresponding phenotypes. Here, the formation of beige adipocyte phenotypes following treatment with Cy3G was evaluated using 3T3-L1 adipocytes. Cy3G induced phenotypic changes to white adipocytes, such as increased multilocular lipid droplets and mitochondrial content. Additionally, the expression of mitochondrial genes (*TFAM*, *SOD2*, *UCP-1*, and *UCP-2*), UCP-1 protein, and beige adipocyte markers (*CITED1* and *TBX1*) in 3T3-L1 adipocytes were increased by Cy3G. Furthermore, Cy3G promoted preadipocyte differentiation by up-regulating of *C/EBPβ* through the elevation of the intracellular cAMP levels. These results indicated that Cy3G elevates the intracellular cAMP levels that induces beige adipocyte phenotypes. This is the first report on the effect of Cy3G on induction of differentiation of

preadipocytes into beige adipocyte phenotypes.

Keywords: Cyanidin-3-glucoside, 3T3-L1 adipocytes, mitochondria, beige adipocyte, differentiation

1. Introduction

The number of overweight and obese individuals has been increasing annually worldwide, resulting in higher prevalence of risk factors for the onset of several diseases such as heart disease, diabetes, stroke, and cancer [1]. When energy intake exceeds energy consumption due to overeating, high-calorie intake, sedentary lifestyle, and aging, the outcome is excessive fat accumulation (increase in the number and/or size of adipocytes) [2, 3]. In addition, obese individuals have decreased metabolic activity [4]. The mitochondria play a significant role in energy generation via ATP production and thermogenesis (dissipation of energy as heat). However, mitochondrial number and activity in adipocytes are decreased by increased levels of blood free fatty acids (FFA) and inflammatory cytokines in obese and diabetic patients [4]. Therefore, it is believed that increasing an individual's metabolic activity is an effective strategy for the prevention and management of medical complications associated with obesity.

The adipose tissue plays an important role in the regulation of energy balance and homeostasis. In mammals, the adipose tissue is classified into two types, white adipose tissues (WAT) and brown adipose tissues (BAT) [5]. WAT stores excess energy as triglycerides and become bigger and more numerous with weight gain [5]. WAT regulates energy homeostasis via its endocrine functions. Morphologically,

white adipocytes contain unilocular, large lipid droplets and fewer mitochondria [5].

In obese individuals, oxygen consumption, thermogenic activity, and mitochondrial content of white adipocytes were decreased [6]. In contrast, BAT, which plays an important role in metabolism, is specialized to perform thermogenesis [7]. Morphologically, brown adipocytes contain multilocular lipid droplets, numerous mitochondria, and express uncoupling protein-1 (UCP-1) [7]. UCP-1, which is localized to the inner membrane of mitochondria, generates heat instead of ATP by diminishing the proton gradient [8]. BAT ablation or dysfunction in rodents caused obesity by decreasing energy expenditure [9]. On the other hand, enhancement of BAT function and number was effective against obesity by increasing energy expenditure [10]. Nevertheless, compared with WAT depots, BAT depots in human adults are much smaller and present in defined places, such as the neck and supraclavicular region [9]. Additionally, BAT activity and mass decline with aging [11].

Brown-like adipocytes, which contain multilocular lipid droplets and express UCP-1, appear in WAT [7]. These cells are called beige or **brite** (brown in white) adipocytes [7, 12]. Beige adipocytes not only have characteristics similar to brown adipocytes, such as UCP-1 expression, thermogenesis, and higher numbers of mitochondria compared to white adipocytes, but also express several beige adipocyte

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3 markers, such as CD137, T-box transcriptional factor (TBX1), transmembrane protein
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6 26 (TMEM26), and Cbp/p300-interacting transactivator 1 (CITED1) [7, 8]. Brown
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9 and beige adipocytes can contribute to the regulation of whole-body energy expenditure
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12 [8]. Therefore, an increase in the number or activity level of beige adipocytes in WAT
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15 is effective for maintaining good health or prevention of obesity and other metabolic
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18 diseases [8]. It has been established that external stimulations such as exercise,
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21 chronic cold exposure, norepinephrine, irisin, and PPAR γ agonist can induce formation
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24 of beige adipocytes [13]. Additionally, recent studies have reported that several
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27 dietary compounds, such as fucoxanthin and capsaicin, could induce formation of beige
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30 adipocytes [14].
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35 Cyanidin-3-glucoside (Cy3G), which is a typical polyphenolic anthocyanin
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38 compound, is a pigment found in numerous colorful fruits and vegetables, such as black
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41 soybeans, blueberries, and grapes [15]. Several studies revealed that Cy3G has many
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44 positive health effects, including anti-oxidative [16] and anti-diabetic effects [17].
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47 Furthermore, pharmacokinetic studies revealed that absorbed Cy3G reaches not only the
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50 plasma but also the adipose tissues in glycosidic form [18, 19]. Therefore, it is
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53 predicted that Cy3G could be an effective treatment for the prevention or treatment of
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56 obesity. We have recently reported that Cy3G can induce differentiation of
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pre-adipocytes into smaller lipid droplets through the up-regulation of the peroxisome proliferator-activated receptor γ (PPAR γ) and CCAAT/enhancer binding protein α (C/EBP α) in 3T3-L1 adipocytes [17]. PPAR γ and C/EBP α are involved in the maintenance of stable differentiation of both white and brown adipocytes [9]. Additionally, multilocular lipid droplets is one of the hallmarks of beige adipocytes [7]. However, since the effects of Cy3G on the induction of beige adipocytes and its phenotypes have not yet been reported, here, we investigated the effect of Cy3G on induction of beige adipocyte phenotypes using the 3T3-L1 cell line as white adipocytes cell model to gain a better understanding of its effect on obesity.

2. Materials & Methods

2.1. Chemicals

Cy3G, Rhodamine 123, and Rosiglitazone were purchased from Wako (Tokyo, Japan).

HBSS was purchased from Gibco (NY, USA). DMEM, HEPES, Triton X-100,

Radio-Immunoprecipitation Assay (RIPA) buffer, protease inhibitor cocktail, β -Actin

antibody, 3-isobutyl-1-methylxanthine (IBMX), and Ro 20-1724 were purchased from

Sigma (MO, USA). 3- (4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

(MTT) and Hoechst 33342 solution were purchased from Dojindo (Kumamoto, Japan).

2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Enzo Life

Sciences (Lausen, Switzerland). UCP-1 antibody was purchased from Abcam

(Cambridge, UK). PGC-1 α (3G6), AMPK α 1 and phospho-AMPK α (Thr172)

antibodies were purchased from Cell Signaling Technology (Hertfordshire, UK).

2.2. Cell culture and differentiation

The mouse 3T3-L1 cells (JCRB Cell Bank, Osaka, Japan) were cultured in DMEM

supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin (5000

μ g/ml) – streptomycin (5000 IU/ml) (Lonza, Japan) at 37 °C in a humidified atmosphere

of 5% CO₂. Adipocyte differentiation and Oil Red O staining were performed using an Adipogenesis Assay kit (Cayman Chemical Co., Ann Arbor, USA) following the manufacturer's instructions. Briefly, for differentiation into mature adipocytes, 3T3-L1 cells were seeded at 3×10^4 cells/cm² and cultured to reach confluence. Two days post-confluence (Day 0), cells were treated with differentiation hormonal cocktail (DMEM containing DEX, IBMX and insulin) for 72 h (Day 3) and transferred to DMEM containing insulin that was changed every 2 days for 4 days until more than 80% of cells were differentiated (Day 7). After differentiation, cells were stained with Oil Red O. During the differentiation process (Day 0 to Day 7), 3T3-L1 cells were treated with or without Cy3G (Fig. 1 A).

2.3. Cell proliferation (MTT assay)

Differentiated 3T3-L1 adipocytes (Day 7) were incubated with MTT solution (5 mg/ml) for 3 h or until formazan crystals were formed which were then dissolved by adding 10% sodium dodecyl sulfate (Wako, Japan) followed by overnight incubation (around 16 h). After that, the absorbance was detected at 570 nm using a Powerscan HT plate reader (Dainippon Sumitomo Pharma Co, Ltd., Japan). The values were normalized to the value of the growth medium and calculated as a percentage (%) of control.

2.4. Measurement of the mitochondrial content by Rhodamine 123

Differentiated 3T3-L1 adipocytes (Day 7) were incubated with a fluorescent dye Rhodamine 123 (10 µg/ml) in 10 mM HEPES-HBSS buffer (pH 7.4) for 20 min at 37°C. Fluorescent images were obtained using a BZ-X710 All-in-One fluorescence microscope (Keyence, Osaka, Japan). To quantify the rhodamine 123 content, cells were lysed using 1% Triton X-100 and the fluorescence intensity (excitation/emission 485/528 nm) was measured using a Powerscan HT plate reader. The rhodamine 123 content was calculated using the standard curve and values were calculated as a percentage (%) of control.

2.5. Real-time PCR analysis

Total RNA isolation and TaqMan real-time PCR amplification reactions were performed as previously described [17]. For the quantification of gene expression, the following TaqMan probes purchased from Applied Biosystems (CA, USA) were used: *β-actin* (Mm00607939_s1), *FABP4* (Mm00445878_m1), *TFAM* (Mm00447485_m1), *CytC* (Mm01621048_s1), *PDK4* (Mm01166879_m1), *SOD2* (Mm01313000_m1), *UCP-1* (Mm01244861_m1), *UCP-2* (Mm00627599_m1), *C/EBPβ* (Mm00843434_s1), *TBX1*

(Mm00448949_m1), *CITED1* (Mm01235642_g1), *PPAR γ* (Mm01184322_m1), and *C/EBP α* (Mm00514283_s1). The PCR amplification cycles were as follows: 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of PCR (95°C, 15 sec; 60°C, 60 sec). The mRNA levels of all genes were normalized to the *β -actin* level (internal control).

2.6. Measurement of intracellular ATP level

The intracellular ATP level in 3T3-L1 adipocytes were measured using “Cellno” ATP ASSAY reagent (TOYO Ink, Japan) according to the manufacturer’s instructions. Briefly, ATP assay reagents were added to each well (100 μ l/well) of differentiated cells (Day 7), incubated for 10 min at 25°C, and the luminescence was measured using a Powerscan HT plate reader. The values were calculated as a percentage (%) of control.

2.7. Measurement of intracellular ROS level

Intracellular reactive oxygen species (ROS) level was measured using a fluorescent dye, DCFH-DA. Differentiated cells (Day 7) were cultured in serum-free DMEM containing 10 μ M DCFH-DA at 37°C for 30 min. Then, fluorescence intensity (excitation/emission 485/528 nm) was measured using a Powerscan HT plate reader. The values were calculated as a percentage (%) of control.

2.8. Western Blotting

Total protein was extracted using RIPA buffer containing a protease inhibitor cocktail according to the manufacturer's instructions. Protein samples (15 µg) were separated using 10 % SDS-PAGE and transferred to a PVDF membrane (Merck Millipore, USA). Membranes were incubated with primary antibody at 4°C overnight, then washed and incubated with secondary antibodies, IRDye 800CW Donkey anti-rabbit IgG or IRDye 680LT Goat anti-mouse (LI-COR, Inc., NE, USA), at room temperature for 30 min. The signal was detected using the OdysseyFc Imaging System (LI-COR, Inc., NE, USA). All protein quantifications were normalized to the β-Actin expression level.

2.9. Measurement of intracellular cAMP level

The intracellular cAMP level in 3T3-L1 cells were measured using the cAMP-GloTM MAX assay (Promega, USA) according to the manufacturer's instructions. Briefly, 3T3-L1 cells were seeded and cultured to reach confluence in white, clear-bottom tissue culture 96 well plates (Corning, NY, USA). Two days post-confluence (Day 0), cells were cultured in serum-free DMEM containing 20 mM MgCl₂ with or without phosphodiesterase (PDE) inhibitors (500 µM IBMX and 100 µM Ro20-1724) and with

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3 or without Cy3G (100 μ M) for 30 min. Then, the luminescence was measured using a
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6 Powerscan HT plate reader. Intracellular cAMP levels were calculated based on a
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9 standard curve and values were expressed as a percentage (%) of control. In this assay,
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13 PDE inhibitors were used to prevent cAMP hydrolysis during the assay.
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19 **2.10. Statistical analysis**

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22 All the results were expressed as the mean $\pm$ standard deviation (SD), and the statistical
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25 evaluation was performed using Student's t-test when two value sets were compared.
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29 When multiple comparisons were carried out, one way analysis of variance (ANOVA)
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32 followed by Tukey's multiple comparison test was performed using SPSS (IBM
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35 Statistics for Windows, version 22.0. IBM Corp, Armonk, NY). $P < 0.05$ was
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38 considered to be statistically significant.
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3. Results

3.1. Cy3G induced multilocular lipid droplets in adipocytes by promoting differentiation

To evaluate the effect of adipocyte differentiation, 3T3-L1 preadipocytes were treated with different concentrations of Cy3G (20, 40, 60, 80, and 100 μ M) during the differentiation process (Day 0 to Day 7) (Fig. 1A). As shown in Fig. 1 B, Cy3G treatment (20 to 100 μ M) was not cytotoxic to 3T3-L1 adipocytes. Moreover, Cy3G-treated 3T3-L1 adipocytes formed multilocular lipid droplets (Fig. 1 D). As a result of adipocyte differentiation, the expression of adipocytes markers, such as *FABP4* (also known as aP2), *GLUT4*, and adiponectin, are reportedly up-regulated by *PPAR γ* and *C/EBP α* , an important transcriptional factor that regulates adipocyte differentiation [20]. In our previous study, we revealed that Cy3G increased the expression of *PPAR γ* and *C/EBP α* expression [17]. As shown in Fig. 1 C, Cy3G treatment of 50 μ M and 100 μ M increased *FABP4* expression by 1.3 fold and 2.4 fold, respectively. Therefore, these results suggest that Cy3G induced multilocular lipid droplet in adipocytes by promoting differentiation.

3.2. Cy3G increased the mitochondrial content and mitochondrial genes expression through AMPK activation

Increased mitochondrial number and the expression of mitochondrial genes, especially UCP-1, are known markers of brown and beige adipocytes [7]. Therefore, the effect of Cy3G on mitochondrial content and the expression levels of mitochondrial genes of 3T3-L1 adipocytes were evaluated. Rhodamine 123 was used as a fluorescent probe to stain mitochondria [21]. As shown in Fig. 2 A and B, Cy3G increased the number of mitochondria as shown by the increase in rhodamine 123 content in 3T3-L1 adipocytes to 114% and 119%, respectively. The AMP-activated protein kinase (AMPK) pathway, which acts as a sensor of cellular energy status, promotes mitochondrial biogenesis, glucose uptake, and β -oxidation [22]. Mitochondrial transcriptional factor A (TFAM) is known as a key regulator of mitochondrial biogenesis, and exerts control over replication and transcription of the mitochondrial genomes [23]. As shown in Fig. 2 C, Cy3G treatments of 50 and 100 μ M increased phosphorylation of AMPK to 1.3 and 1.4 fold, respectively. Furthermore, the expression of *TFAM* and mitochondrial genes (*CytC*, *PDK4*, *UCP-1*, *UCP-2*, and *SOD2*) were up-regulated (Fig. 2 D). These results

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3 indicate that Cy3G increased mitochondrial content and the expression of mitochondrial
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6 genes through AMPK activation.
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10 11 12 **3.3. Cy3G induced similar characteristics of brown adipocytes and the expression** 13 14 **of beige adipocyte markers** 15 16

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22 Beige adipocytes have characteristics similar to brown adipocytes, such as UCP-1 and
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25 PGC-1 α expression [7, 8]. As shown in Fig. 2 E, Cy3G increased UCP-1 and PGC-1 α
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28 protein expression by 2.1- and 1.7-fold, respectively. UCP-1 plays an important role in
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31 thermogenesis and decreasing the production of ROS by diminishing the proton
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34 gradient instead of ATP synthesis [24]. Additionally, Cy3G-treated 3T3-L1 adipocytes
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37 showed a decline in intracellular ROS and ATP production (Fig. 3 A and B). *C/EBP β*
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40 is more highly expressed in brown adipocytes and regulates gain of brown adipocyte
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43 phenotypes, such as UCP-1 expression [9, 10, 25]. In the present study, *C/EBP β*
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46 expression was up-regulated by Cy3G (Fig. 3 C). These results indicated that Cy3G
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49 induced similar characteristics of brown adipocyte in 3T3-L1 adipocytes. UCP-1
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52 positive beige adipocytes selectively expressed *CITED1* [26]. As shown in Fig. 3 D,
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57 Cy3G increased the expression of beige adipocyte selective markers, *TBX1* and *CITED1*.
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Therefore, these results suggest that Cy3G induced beige adipocyte phenotypes in 3T3-L1 adipocytes.

3.4. Cy3G induced the expression of beige adipocyte markers by regulating the preadipocytes differentiation

Chronic PPAR γ agonist treatment has been shown to increase brown and beige adipocytes-associated genes expression in primary adipocytes differentiated from preadipocytes, it suggesting that the regulation of differentiation of preadipocytes is important step for the development of brown and beige adipocyte phenotypes in mature adipocytes [27]. In the present study, Cy3G up-regulated *TBX1* and *CITED1* expression in 3T3-L1 cells after 3 days of treatment (Fig. 4). *CITED1* expression was especially up-regulated at Day 3 (Fig. 4). PPAR γ and C/EBP α are important transcriptional factors that regulate both white and brown adipocytes differentiation [12, 28]. During the adipocyte differentiation process, C/EBP α expression is enhanced in response to treatment with a cocktail of differentiation hormones (DEX, IBMX, and insulin) for 2-3 days [29]. As shown in Fig. 5, Cy3G increased the expression of PPAR γ and C/EBP α both in the presence and in the absence of differentiation cocktail at

3 days of induction (Day 3). Therefore, these results suggest that Cy3G promoted the expression of beige adipocyte markers by regulating the preadipocytes differentiation.

3.5. Cy3G increased *C/EBPβ* expression by increasing the intracellular cAMP levels through inhibition of PDE

The transcription of *PPARγ* and *C/EBPα* is induced by *C/EBPβ*, a widely recognized and important transcriptional factor for preadipocytes differentiation [28, 29]. *C/EBPβ* expression is rapidly and transiently increased by treatment with a cocktail of differentiation hormones [28, 29]. In our present study, Cy3G significantly increased *C/EBPβ* expression by 1.9 fold for 1 h treatment and 3.2 fold for 3 h treatment, respectively (Fig. 6 A). Additionally, Cy3G up-regulated *C/EBPβ* expression by 1.8 fold in the absence of the differentiation hormonal cocktail (Fig. 6 B). Intracellular cAMP levels are known to regulate the expression of *C/EBPβ* [30], and as shown in Fig. 6 C, Cy3G increased the intracellular cAMP level to 138 %. In the absence of PDE inhibitors (IBMX and Ro 20-1724), intracellular cAMP levels were significantly lower than those of the Cy3G (PDE inhibitor +), indicating considerable cAMP hydrolysis (Fig. 6 C). Therefore, these results indicate that Cy3G increased *C/EBPβ* expression

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3 by increasing the intracellular cAMP levels through inhibition of PDE.
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9 10 **4. Discussion**

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12 Obesity is a serious metabolic disorder that can contribute to the onset of
13 several diseases such as diabetes, hypertension, and arteriosclerosis [1]. Therefore, the
14 prevention and improvement of obesity is important for attaining and maintaining good
15 health. Increased metabolic activity is effective for the prevention and improvement of
16 obesity. Beige adipocytes are similar to brown adipocytes in terms of thermogenic
17 activity, UCP-1 expression, and high number of mitochondria [7, 14]. Therefore, the
18 increase in number and activity of beige adipocytes induced by pharmacological and
19 dietary compounds has gained attention as an effective means to fight obesity [14].
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38 Cy3G, a polyphenolic compound, is present in high amounts in black soybeans,
39 blueberries, and grapes [15]. Pharmacokinetic studies revealed that administered
40 Cy3G is absorbed by adipose tissue in its unchanged glycosylated form, clearly indicating
41 Cy3G can reach adipose tissue through the circulation systems [18, 31]. In our present
42 study, we revealed that Cy3G induced the adipocytes to have not only characteristics
43 similar to brown adipocyte, such as an increase of multilocular lipid droplets,
44 mitochondrial content, and UCP-1 expression, but also the ability to express beige
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3 adipocyte-associated markers in 3T3-L1 adipocytes (Fig. 1-3). 3T3-L1 adipocytes are
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6 a well-studied *in vitro* model of white adipocytes. The results of this study showed
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9 that Cy3G induced the differentiation of 3T3-L1 adipocytes into beige adipocyte
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12 phenotypes which suggest the possibility of improving obesity through Cy3G
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15 administration [17, 32].
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19 Obese individuals have increased mitochondrial ROS production, causing the
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22 synthesis of faulty proteins, oxidized lipids, and mtDNA mutations, which are related to
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25 mitochondrial dysfunction, decreased metabolic activity, and cellular insulin sensitivity
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28 [33]. In this study, we found that Cy3G decreased the intracellular ROS level as
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31 indicated by the changes in the expression level of adipocyte markers associated with
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34 ROS production in mitochondria (Fig. 2 D and 3 A). UCP-1, a marker of brown and
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37 beige adipocytes, plays an important role in thermogenesis and decreases the ROS
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40 produced by the mitochondria [24]. UCP-2, which is a mitochondrial anion carrier
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43 present in the mitochondrial inner membrane of WAT, also controls ROS production in
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46 the mitochondria, protecting against oxidative stress [34]. Additionally, ROS
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49 scavenging enzymes, such as superoxide dismutase (SOD), is involved in the regulation
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52 of intracellular ROS levels [33]. Therefore, the results shown in Fig. 2 D and 3 A
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3 increasing of *UCP-1*, *UCP-2*, and *SOD2* expression. It has been established that
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6 increased ROS production induces insulin resistance, a key pathological feature of
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9 T2DM, through the activation of c-Jun N-terminal kinase and I κ B kinase [35]. In our
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12 previous study, we have shown that Cy3G can ameliorate T2DM by increasing the
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15 insulin sensitivity of adipocytes [17]. Therefore, it is predicted that decreased the
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18 intracellular ROS production following Cy3G treatment can also be attributed to
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22 Cy3G-induced activation of insulin sensitivity.
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25 UCP-1 dissipates the proton gradient of the mitochondrial membrane to
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28 produce heat (thermogenesis) instead of ATP [8]. It has also been established that
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31 overexpression of UCP-1 in adipocytes leads to an increase in the AMP/ATP ratio and
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34 activation of AMPK [36]. Therefore, our findings, that decreased intracellular ATP
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37 production in Cy3G-treated 3T3-L1 adipocytes (Fig. 3 B) is consistent with what is
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40 known about UCP-1 activity. AMPK pathway, which is activated in response to
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43 depleted ATP levels, promotes mitochondrial biogenesis, glucose uptake, and
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46 β -oxidation [22]. In addition to increasing mitochondrial content, Cy3G increased
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49 AMPK phosphorylation and the expression of *TFAM* (Fig. 2). *TFAM* is a key
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52 regulator of mitochondrial biogenesis [23]. Therefore, it is suggested that increasing
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55 of mitochondrial content is regulated by *TFAM* up-regulation through the AMPK
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3 pathway. Some AMPK activators, such as resveratrol and curcumin, can induce
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6 brown-like adipocytes [37, 38]. Therefore, it is most likely that the activation of
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9 AMPK in response to mitochondrial uncoupling explains the increase of mitochondria
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12 content in Cy3G-treated adipocytes.
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16 Increased mitochondrial number is one of the hallmarks of beige adipocytes [7].
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18 Mitochondrial biogenesis and oxidative activity play a significant role in adipocyte
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21 differentiation because the adipocyte differentiation process requires a large amount of
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24 ATP during this process [39]. Therefore, with adipocyte differentiation, mitochondrial
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27 biogenesis and oxidative capacity are induced [39, 40]. Actually, PPAR γ agonists,
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30 such as rosiglitazone and pioglitazone, increase the mitochondrial genes and proteins
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33 expression accompanied by an increasing in the rate of adipocyte differentiation [41].
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36 In the present study, Cy3G increases mitochondrial content as well as mitochondrial
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39 oxidative genes expression, such as *CytC* and *PDK4*, in 3T3-L1 adipocytes (Fig. 2).
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42 These results suggest that Cy3G not only increases mitochondrial biogenesis and
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45 oxidative capacity, but also promotes adipocyte differentiation.
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51 Differentiation of preadipocytes into mature adipocytes is associated with the
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54 regulation of adipokine secretion, insulin sensitivity, and metabolic activity [20].
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57 PPAR γ and C/EBP α are transcription factors for numerous adipocytes-related genes and
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3 protein expression, and are involved in both white and brown adipocytes differentiation
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6 [12, 20]. Chronic PPAR γ agonist treatment or prolonged triiodothyronine, IBMX, and
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9 rosiglitazone treatment when preadipocytes are differentiating into mature adipocytes
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12 can result to increase in brown and beige adipocytes-associated genes expression in
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15 mature adipocytes, suggesting that the regulation of differentiation of preadipocytes is
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18 important step for the development of brown and beige adipocytes phenotypes in
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21 mature adipocytes [27, 42, 43]. In this study, we observed that Cy3G also promoted
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24 the expression of beige adipocyte markers by regulating preadipocytes differentiation
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27 (Fig. 4-6). Therefore, it is clear that regulating the preadipocytes differentiation may
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30 be an important step for enhancing the production of beige adipocyte phenotypes in
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33 mature adipocytes. PPAR γ agonists, which can promote preadipocytes differentiation,
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36 induce the formation of beige and brown adipocytes phenotypes by stabilizing the PR
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39 domain-containing protein-16 (PRDM16) and increasing early B-cell factor-2 (EBF2)
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42 expression [27, 44]. Cy3G, as we have previously reported, does not have PPAR γ
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45 agonist activity [17]. Therefore, it is predicted that Cy3G induces the formation of
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48 beige adipocytes phenotypes through a different mechanism.
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54 C/EBP β is also widely recognized as an important transcriptional factor for
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57 preadipocytes differentiation, as it induces the transcription of PPAR γ and C/EBP α [30].
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In committed preadipocytes, *C/EBPβ* expression is very low but it is transiently enhanced by adipogenic stimulation, specifically via cAMP stimulation [30]. Therefore, increasing *C/EBPβ* expression and elevation of intracellular cAMP levels by Cy3G are indicators of Cy3G promoted preadipocyte differentiation (Fig. 6). The cAMP-responsive element-binding protein (CREB) is a known as central transcriptional activator of the adipocyte differentiation, are activated by cAMP. Activated CREB induces expression of *C/EBPβ* [45, 46]. As shown in Fig. 6, Cy3G increased intracellular cAMP levels (30 min treatment) and *C/EBPβ* expression (1 h treatment). Therefore, we suggest that increasing intracellular cAMP levels up-regulate *C/EBPβ* expression. In the present study, *C/EBPβ* expression was up-regulated by Cy3G in both the early phase (1 h and 3 h) and the mature stage (Day 7) (Fig. 3 C and 6). *C/EBPβ* has play an important role in regulating the formation of the brown adipocyte phenotype [10, 25]. *C/EBPβ* induces the expression of PGC-1 α and UCP-1 by interacting with PR domain- containing protein-16 during early differentiation and in combination with cAMP stimulation, is able to induce white preadipocyte 3T3-L1 cells to a brown-like adipocytes [10, 25, 43, 47]. In this study, we found that Cy3G increased PGC-1 α and UCP-1 expression and intracellular cAMP levels (Fig. 2 E and 6 C) which suggests that Cy3G-increased cAMP levels can promote the transcription of

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3 beige adipocytes-related genes, therefore inducing the differentiation of white
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6 preadipocyte 3T3-L1 cells into beige adipocytes (Fig. 7). It is known that activated
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9 apoptosis signal-regulating kinase 1 (ASK1)-p38 axis in response to cAMP also induces
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12 brown and beige adipocyte function such as UCP-1 expression [48]. Therefore, we
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15 anticipate that ASK1 signalling might be involved in inducing of beige adipocyte
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18 phenotypes by Cy3G.
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22 PDEs are key enzymes for regulating intracellular cAMP and cGMP levels.
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25 According to Merlin J et al [13], elevated intracellular cAMP levels and inhibitor of
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28 PDEs, which induce UCP-1 expression in BAT and iWAT. In the present study, Cy3G
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31 increased the intracellular cAMP level by inhibiting PDE (Fig. 6 C). Based on in vitro
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34 cell-free assay results rather than a cellular model assay, Cy3G showed PDE inhibitory
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37 effect [49]. Therefore, we suggest that Cy3G increases the intracellular cAMP levels
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40 through PDE inhibition.
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48 In conclusion, the present study revealed that Cy3G induces the 3T3-L1
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51 adipocytes to obtain not only similar characteristics as the brown adipocytes, such as
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54 increased multilocular lipid droplets, mitochondrial content, and UCP-1 and PGC-1 α
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57 expression, but also those of the beige adipocyte markers. Additionally, Cy3G
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3 promoted preadipocytes differentiation by up-regulating of *C/EBPβ* through the
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6 elevation of the intracellular cAMP levels. These results indicated that increased
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9 cAMP levels through Cy3G treatment can induce the transcription of beige
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12 adipocytes-related genes and promote white preadipocyte 3T3-L1 cells differentiation
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15 into beige adipocytes (Fig. 7). This is the first report on the effect of Cy3G on
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18 induction of differentiation of preadipocytes into beige adipocyte phenotypes. The
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21 results of our study suggest that Cy3G may be used as an effective therapeutic agent for
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24 prevention and treatment of obesity and obesity-related metabolic diseases.
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31 **Conflict of interest statement**

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35 The authors indicated no conflicts of interest.
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41 **Acknowledgment**

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Figure legends

Figure 1. Effect of Cy3G on lipid droplet morphology and adipocyte differentiation in 3T3-L1 adipocytes.

Experimental procedure of differentiation of 3T3-L1 adipocytes. During the differentiation process (Day 0 to Day 7), 3T3-L1 cells were treated with or without 20 to 100 μ M Cy3G (A). After differentiation, cell viability (B), *FABP4* mRNA levels (C), and lipid droplet morphology (D) were evaluated. (B) Cell viability was determined by MTT assay. Values are expressed as a percentage (%) of the control. (C) *FABP4* mRNA levels were normalized to β -actin expression levels. (B and C) Results are expressed as the mean $\pm$ SD of triplicate experiments. * $P < 0.05$, **

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3 $P < 0.01$ indicates the mean value is significantly different from that of the control. (D)

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6 Differentiated 3T3-L1 adipocytes were stained by Oil Red O. Photographs are on the
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8 Control (Left) and 100 μ M Cy3G-treated 3T3-L1 adipocytes (Right). Scale bar
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10 indicates 50 μ m.
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19 **Figure 2. Effect of Cy3G on mitochondrial content and the expression of**
20 **mitochondrial genes and UCP-1 in 3T3-L1 adipocytes.**
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25 3T3-L1 preadipocytes were treated with or without 50 and 100 μ M Cy3G during the
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27 differentiation process for 7 days. After differentiation, mitochondrial content (A, B),
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29 phosphorylation of AMPK (C), mitochondrial gene (*TFAM*, *CytC*, *PDK4*, *SOD2*, *UCP-1*,
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31 and *UCP-2*) expression (D), and UCP-1 and PGC-1 α protein expression (E) in 3T3-L1
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33 adipocytes were evaluated. (A, B) Mitochondria were stained by a mitochondrial
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35 specific dye, Rhodamine 123. (A) Photographs are on the Control (Left) and 100 μ M
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37 Cy3G-treated 3T3-L1 adipocytes (Right). Rhodamine 123 (mitochondria) is green and
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39 Hoechst 33342 (nuclei) is blue. Scale bar indicates 100 μ m. (B) To quantify the
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41 rhodamine 123 content, cells were lysed using 1% Triton X-100 and fluorescence
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43 intensity (485 nm/528 nm) was measured. Values were expressed as a percentage (%)
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45 of the control. (C and E) Protein expression levels were normalized to the β -actin
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3 expression levels. (D) Expression levels of mRNA were normalized to the *β-actin*
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6 expression level. (B and E) Rosiglitazone (1 μM) is used as a positive control.
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9 Results are expressed as the mean ± SD of triplicate experiments. * *P*<0.05, **
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13 *P*<0.01 indicates the mean value is significantly different from that of the control.
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19 **Figure 3. Effect of Cy3G on intracellular ROS, ATP production, and the expression**
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21 **of beige adipocyte markers in 3T3-L1 adipocytes.**
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25 3T3-L1 preadipocytes were treated with or without 50 and 100 μM Cy3G during the
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28 differentiation process for 7 days. After differentiation, intracellular ROS production
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31 (A), intracellular ATP production (B), and gene expression of *C/EBPβ* (C) and beige
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34 adipocyte markers (D) in 3T3-L1 adipocytes were evaluated. (A and B) Values were
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37 expressed as a percentage (%) of the control. (C and D) Values were normalized to the
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40 *β-actin* expression levels. Results are expressed as the mean ± SD of triplicate
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43 experiments. * *P*<0.05, ** *P*<0.01 indicates the mean value is significantly different
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47 from that of the control.
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54 **Figure 4. Time dependent effect of Cy3G on *TBX1* and *CITED1* expression in**
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57 **3T3-L1 cells.**
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3T3-L1 preadipocytes were treated with or without 50 and 100 μ M Cy3G during the differentiation process for 3, 5, and 7 days. After treatment, the gene expression of *TBX1* (A) and *CITED1* (B) in 3T3-L1 cells were evaluated using real-time PCR. (A and B) Values were normalized to the β -actin expression levels and expressed relative to the undifferentiated 3T3-L1 cells. Results are expressed as the mean $\pm$ SD of triplicate experiments. * $P < 0.05$, ** $P < 0.01$ indicates the mean value is significantly different from that of the control.

Figure 5. Effect of Cy3G on *PPAR γ* and *C/EBP α* expression in 3T3-L1 cells.

3T3-L1 preadipocytes (Day 0) were treated with or without Cy3G (100 μ M) in the presence of the differentiation hormonal cocktail (A) or the absence of the differentiation hormonal cocktail (B) for 3 days. And then, gene expression of levels of *PPAR γ* and *C/EBP α* were evaluated using real-time PCR and normalized to the β -actin expression levels. Results are expressed as the mean $\pm$ SD of triplicate experiments. ** $P < 0.01$ indicate the mean value that is significantly different from that of each control groups.

Figure 6. Effect of Cy3G on early phase of adipocyte differentiation in 3T3-L1

cells.

(A) 3T3-L1 preadipocytes (Day 0) were treated with or without Cy3G (100 μ M) in the presence of a differentiation hormonal cocktail for 1-3 h. (B) 3T3-L1 preadipocytes were treated with or without 100 μ M Cy3G in the absence of the differentiation hormonal cocktail for 1 h. Expression levels of *C/EBP β* mRNA were evaluated and normalized to the *β -actin* expression levels. ** $P < 0.01$ indicates significant difference compared to the control. (C) 3T3-L1 preadipocytes were treated with or without Cy3G (100 μ M) and with or without phosphodiesterase (PDE) inhibitor (500 μ M IBMX and 100 μ M Ro20-1724) for 30 min. After that, the intracellular cAMP level was measured. Values were calculated using the standard curve, then quantified and expressed as a percentage (%) of the control. Results are expressed as the mean $\pm$ SD of triplicate experiments. ** $P < 0.01$ indicates significant difference compared to the Control (PDE inhibitor +). ## $P < 0.01$ indicates significant difference compared to the Cy3G (PDE inhibitor +). Multiple comparisons were carried out using ANOVA followed by Tukey's multiple comparison test.

Figure 7. Suggested pathway for cyanidin-3-glucoside induced beige adipocyte phenotypes by promoting adipocyte differentiation in 3T3-L1 adipocytes

Cy3G induced beige adipocytes phenotypes, such as increased mitochondrial content and the expression of mitochondrial genes, UCP-1 expression, and beige adipocytes markers by promoting adipocyte differentiation through *C/EBP β* up-regulation. This *C/EBP β* up-regulation was induced by increasing the intracellular cAMP level.

Figure 1

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Figure1.

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Figure 2

Figure 2.

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