Effect of Food-derived Compound Cyanidin-3-glucoside on Improvement of Metabolic Environment

January 2017

Toshiya MATSUKAWA

Effect of Food-derived Compound Cyanidin-3-glucoside on Improvement of Metabolic Environment

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Environmental Studies

(Doctoral Program in Sustainable Environmental Studies)

Toshiya MATSUKAWA

Abstract

In modern society, people are predisposed to various diseases because of their lifestyles and environmental factors such as dietary habits, sedentary lifestyle, smoking stress, and exposure to toxic substances. These lifestyle and environmental factors can adversely affect the metabolic environment, such as the balance of metabolism, hormones, and nutrition, eventually resulting in the development of lifestyle-related diseases. The number of patients suffering from lifestyle-related diseases such as obesity, diabetes, hypertension, hyperlipidemia, non-alcoholic steatohepatitis, and cancer is increasing annually worldwide. In fact, more than two thirds of deaths in Japanese populations are reported to be due to lifestyle-related diseases, or "seikatsusyukanbyo," as they are known in Japan. Therefore, prevention and amelioration of lifestyle-related diseases by regulating the metabolic environment is important for human health. Recently, nutraceuticals, such as dietary fiber, probiotics, vitamins, and polyphenols, have gained attention as alternatives to conventional pharmaceuticals, since they promote the proper maintenance of a healthy metabolic environment via the regulation of both energy and hormone balance. Cyanidin-3-glucoside (Cy3G), a polyphenol compound present in numerous colorful fruits and vegetables such as black soybean, blueberry, and grapes. In a previous study, Cy3G was shown to increase the expression of *peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α)*, encoding a transcriptional coactivator essential for the regulation of metabolism, in skeletal muscle cells. It has been suggested that PGC-1α is an important regulator of exercise adaptation in skeletal muscle and prevent metabolic diseases. In this study, it was hypothesized that Cy3G is effective for enhancing exercise performance and preventing metabolic disorders by regulating metabolic environments. This thesis investigated the effect and regulatory mechanisms of Cy3G on the metabolic environment using animal experiments and *in vitro* cellular model experiments.

Firstly, the effects of Cy3G on both exercise performance and the metabolic activity of the skeletal muscle were investigated. Regular exercise and physical training contribute to the prevention and alleviation of numerous lifestyle-related diseases, such as obesity, type 2 diabetes, sarcopenia, and cardiovascular disease, as well as enhances physiological capacity in sports by regulating the metabolic environment. It is thought that enhancing the function of skeletal muscle is effective for both the prevention and improvement of metabolic diseases and enhancement of exercise performance. In this thesis, to examine whether Cy3G can enhance exercise performance (swim until exhaustion with a load corresponding to 10% of their body weight), mice were given

Cy3G or water (control) orally for 15 days and were made to perform weight-loaded forced swimming test. As a result, exercise-induced elevation of lactate levels in Cy3G-treated mice was lower than the increase observed in control mice (water-treated mice). Furthermore, Cy3G increased PGC-1α expression in two skeletal muscles (gastrocnemius and biceps femoris). PGC-1α in the skeletal muscle promotes lactate metabolism by promoting expression of lactate dehydrogenase (LDH) B and monocarboxylate transporter 1 (MCT1) and decreasing LDH A expression. Cy3G group had increased expression of *LDH B* and *MCT1* in both muscles. These results suggest that lactate metabolism in the muscles is enhanced in response to Cy3G-induced PGC-1α upregulation. Additionally, from in vitro study using C2C12 myotubes, it has been revealed that *PGC-1α* upregulation by Cy3G was shown to occur via the Calcium/calmodulin-dependent protein kinase kinase (CaMKK) pathway through the elevation of intracellular cAMP levels. These suggest that Cy3G enhances swimming time by increasing skeletal muscle PGC-1α expression through the elevation of cAMP levels.

Next, the effects of Cy3G on the induction of beige adipocytes, and the corresponding effect of this induction on the prevention of metabolic diseases were investigated using a db/db mice (diabetic model mice) and the 3T3-L1 cell line. In individuals with metabolic diseases such as obesity, diabetes, and cardiomyopathy, PGC-1α expression in skeletal muscle is decreased as a result of increased inflammatory adipokines and free fatty acids (FFAs). Therefore, results discussed in above predict that Cy3G may be effective for not only improved exercise performance, but also prevention of metabolic diseases since Cy3G can increase muscle PGC-1α expression. To explore the preventive effect of Cy3G on metabolic disease, db/db mice were given black soybean seed coat extract (BSSCE), which are rich in Cy3G, for 30 days. The oral administration of BSSCE prevented phenotypes associated with the progression of type 2 diabetes mellitus (T2DM), such as the gain of white adipose tissue (WAT) weight and size. Reduced size of WAT is one of the characteristics of beige adipocytes. Recently, brown-like adipocytes known as beige or brite (brown in white) adipocytes, which appear in the WAT, have gained attention, as beige adipocytes can contribute to the regulation of whole-body energy expenditure. Adipocyte differentiation (differentiation of preadipocytes into mature adipocytes) plays an important role in the development of brown and beige adipocyte phenotypes in mature adipocytes. To evaluate the effect of Cy3G on adipocyte differentiation, 3T3-L1 preadipocytes were treated with Cy3G during the differentiation process. *In vitro* studies revealed that Cy3G induces the 3T3-L1 adipocytes to attain beige adipocyte characteristics (multilocular lipid

droplets, increased mitochondrial content, *uncoupling protein-1* (*UCP-1*) expression and beige adipocyte markers). Furthermore, Cy3G promoted preadipocytes differentiation by increasing of *CCAAT/enhancer-binding protein β* (*C/EBPβ*) through the elevation of the intracellular cAMP. These results indicate that elevated cAMP levels by Cy3G induce beige adipocytes phenotypes in 3T3-L1 adipocytes, all of which ultimately prevent the progression of T2DM.

In conclusion, the results presented in this thesis reveal that Cy3G positively regulates metabolic environments by increasing PGC-1α expression via the elevation of intracellular cAMP levels. These effects contribute to both the enhancement of exercise performance and the prevention of metabolic diseases, and indicate that Cy3G may be an effective therapeutic agent for the prevention and/or treatment of lifestyle-related diseases.

Abbreviations and Acronyms

AMPK: AMP-activated protein kinase
ANOVA: Analysis of variance
BAT: Brown adipose tissues
BSSCE: Black soybean seed coat extract
CaMK: Calmodulin kinase
CaMKK: Calcium/calmodulin-dependent protein kinase kinase
C/EBP: CCAAT/enhancer-binding protein
CITED1: Cbp/p300-interacting transactivator 1
CPT-1β: Carnitine palmitoyl transferase-1β
Cy3G: Cyanidin-3-glucoside
DCFH-DA: 2', 7'-Dichlorodihydrofluorescein diacetate
DEX: Dexamethazone
DMEM: Dulbecco's modified eagle medium
DMSO: Dimethyl sulfoxide
EBF2: Early B-cell factor-2
FBS: Fetal bovine serum
ERRα: Estrogen-related receptor α
FABP4: Fatty acid binding protein 4
FFA: Free fatty acids
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GLUT4: Glucose transporter 4
GPDH: Glycerol-3-phosphate dehydrogenase
HS: Horse serum
HSMM: Human Skeletal Muscle Myotubes
IBMX: 3-isobuthyl-1-methylxanthine
IGF-1: Insulin-like growth factor 1
IL-6: Interleukin-6
IR: Insulin receptor
JNK: c-Jun NH_2 terminal kinase
MCP-1: Monocyte Chemoattractant Protein-1
MCT1: Monocarboxylate transporter 1
MTT: 3- (4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NPY: Neuropeptide Y
NRF: Nuclear respiratory factor

PDE: Phosphodiesterase
PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-α
PPARγ: Peroxisome proliferator-activated receptor γ
PPRE: Peroxisome Proliferator Response Elements
PRDM16: PR domain-containing protein-16
ROS: Reactive oxygen species
LDH: Lactate dehydrogenase
LKB1: Liver kinase B1
SD: Standard deviation
SOD: Superoxide dismutase
TBX1: T-box transcriptional factor
TFAM: Mitochondrial transcriptional factor A
TG: Triglycerides
TMEM26: Transmembrane protein 26
TNF-α: Tumor Necrosis Factor-α
TZDs: Thiazolidinediones
T2DM: Type 2 diabetes mellitus
VEGFα: Vascular endothelial growth factor α
UCP: Uncoupling protein
WAT: White adipose tissue

Contents

List of Tables

List of Figures

Publications

Matsukawa T, Inaguma T, Han J, Villareal OM, Isoda H. Cyanidin-3-glucoside derived from black soybeans prevents type 2 diabetes through the induction of differentiation of preadipocytes into smaller and insulin-sensitive adipocytes. J Nutr Biochem 2015; 26: 860-867.

Matsukawa T, Villareal OM, Isoda H. The Type 2 Diabetes-preventive Effect of Cyanidin-3-glucoside on Adipocytes. J Dev Sus Agr 2016; 11: 31-35.

Matsukawa T, Villareal OM, Motojima H, Isoda H. Increasing cAMP levels of preadipocytes by cyanidin-3-glucoside treatment induces the formation of beige phenotypes in 3T3-L1 adipocytes. J Nutr Biochem 2017; 40: 77-85.

Matsukawa T, Motojima H, Sato Y, Takahashi S, Villareal OM, Isoda H. Upregulation of skeletal muscle PGC-1α through the elevation of cyclic AMP levels by Cyanidin-3-glucoside enhances exercise performance. Scientific Reports (*under review*).

Chapter 1

General Introduction

Chapter 1: General Introduction

In modern society, people are predisposed to various diseases because of their lifestyles and environmental factors such as dietary habits, sedentary lifestyle, smoking stress, and exposure to toxic substances. These lifestyle and environmental factors can adversely affect the metabolic environment, such as the balance of metabolism, hormones, and nutrition, eventually resulting in the development of several diseases [1, 2]. Disease occurrence and progression that depend on daily habits and result from the relationship between people and their environment are known as lifestyle-related diseases [1]. The number of patients suffering from lifestyle-related diseases such as obesity, diabetes, hypertension, hyperlipidemia, non-alcoholic steatohepatitis, and cancer is increasing annually worldwide [1]. In Japan, the term "lifestyle-related diseases", also known as "Seikatsusyukanbyo", was proposed in 1996 by the Ministry of Health, Labour and Welfare [2]. More than 60% of deaths in Japan are related to lifestyle-related diseases [2]. Furthermore, diabetes, obesity, and alcoholism can increase the risk of developing other lifestyle-related diseases. Therefore, prevention and amelioration of lifestyle-related diseases by regulating the metabolic environment is important for human health.

Energy unbalance, which occurs when energy intake exceeds energy expenditure, adversely affects the metabolic environment. Not only lifestyle-related factors, such as high-fat diet, overeating, and sedentary lifestyle, but also environmental factors, such as stress, smoking, and pathogens, are involved in the onset of energy unbalance (disturbed metabolic environment), which in turn is related to the onset of lifestyle-related diseases [1, 3, 4]. Enhancing the metabolic rate (increase in energy consumption) in the human body by exercise and activation of metabolic function are effective methods for maintaining the metabolic environment and can prevent lifestyle-related diseases [1, 3, 4]. Metabolic activity is classified into 3 types: basal metabolism, active metabolism, and diet-induced thermogenesis (responsible for 70%, 20%, and 10% of metabolic activity, respectively). Basal metabolism accounts for the highest energy expenditure and contributes to metabolic disease epidemics such as obesity and type 2 diabetes mellitus (T2DM). Numerous tissues, such as skeletal muscle, liver, brain, heart, and adipocytes, are involved in basal metabolism. A decrease in basal metabolism may cause obesity [5]. The mass and metabolic activity of skeletal muscle, which is the main organ of human metabolism (accounting for 40% of basal metabolism) decrease with aging [1, 5]. Therefore, activation of metabolic activity in the skeletal muscle is effective for the prevention of lifestyle-related diseases [1, 3, 4].

Excess energy (calorie) intake leads to fat accumulation in the white adipose

tissues (WAT). The WAT plays an important role in maintaining the metabolic environment by storing and releasing triglycerides (TG) and secreting adipokines (cytokines secreted from adipocytes) [2, 3, 6]. Hypotrophic adipocytes with excessive fat content show decreased adiponectin levels and increased free fatty acids (FFA) and inflammatory adipokines levels, such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6), and monocyte chemotactic protein-1 (MCP-1). These changes lead to lowered metabolism in several tissues [6]. Therefore, enhancing metabolic rate (increase of energy consumption) and regulating fat accumulation and/or secretion of adipokines are effective for maintaining the metabolic environment, which may contribute to the prevention of lifestyle-related diseases [1, 3, 4, 6].

Nutraceuticals include foods such as dietary fiber, probiotics, vitamins, and polyphenols and have recently gained attention as alternatives to pharmaceuticals because of their physiological benefits in preventing diseases and enhancing exercise performance [7]. Polyphenols derived from plants have been reported to function in preventing lifestyle-related diseases by inhibiting fat accumulation or activating metabolism [8, 9]. For instance, isorhamnetin, is present in ginkgo and turnip, prevents fat accumulation in adipocytes [9, 10]. Grape and blueberry contain resveratrol, which can increase metabolism in skeletal muscle [11]. Healthya green tea and coffee, produced by Kao corporation as foods for specified health uses (FOSHU: “Tokuteihokenyoshokuhin”), were suggested to prevent metabolic diseases, as polyphenol compounds present in green tea and coffee have an activating effect on metabolism and prevent the absorption of fats [2, 12, 13, 14]. Furthermore, some polyphenolic compounds attenuate the enhancement of exercise performance and benefits. For instance, supplementation with resveratrol, a polyphenolic compound, can enhance exercise performance by activating metabolic activity and mitochondrial biogenesis in skeletal muscles [15]. Thus, polyphenols may be effective for both prevention of lifestyle-related diseases and enhancement of exercise performance by regulating the metabolic environment as an alternative to pharmaceuticals [7]. Furthermore, enhancing the metabolic rate (increase in energy consumption) in the human body through exercise is effective for preventing lifestyle-related diseases [1, 3, 4]. Polyphenols, which are effective for both lifestyle-related diseases and exercise performance, may have synergistic preventive effects against lifestyle-related diseases.

Anthocyanins, a group of polyphenolic compounds, are present as water-soluble pigments in numerous colorful fruits and vegetables, such as black soybeans, blueberries, and grapes. Daily intake of anthocyanins is estimated to be approximately 180–215 mg in the USA [16]. Cyanidin is the most widespread anthocyanin in nature [17] and exists

in plants in its glycoside form, cyanidin-3-glucoside (Cy3G) (Fig. 1-4). Several studies showed that Cy3G has many positive health effects, such as anti-cancer [18], anti-inflammatory [19], and anti-oxidant effects [20]. Furthermore, pharmacokinetic studies revealed that absorbed Cy3G was detected not only in the plasma but also in several tissues in its glycoside form [21, 22]. Therefore, Cy3G may be effective for preventing lifestyle-related diseases and prolonging human health via its regulatory effects on the metabolic environment. The current research group previously investigated the effect of Cy3G on the metabolism of skeletal muscle using C2C12 myotubes as an *in vitro* model of skeletal muscle [23]. Cy3G increased peroxisome proliferator-activated receptor gamma coactivator 1-α (*PGC-1α*) expression via the calcium/calmodulin-dependent protein kinase kinase (CaMKK) pathway in C2C12 myotubes (Fig. 1-4). Additionally, Cy3G increased mitochondrial content in C2C12 myotubes (Fig. 1-5). PGC-1α is a transcriptional coactivator that plays an important role in the regulation of metabolism [24]. For example, PGC-1α induces mitochondrial biogenesis and mitochondrial genes expression [25]. PGC-1α promotes the transcription of nuclear respiratory factor (NRF) 1 and NRF2, leading to an increase in mitochondrial transcriptional factor A (TFAM), which is leading to mitochondrial DNA replication and mitochondria gene transcription such as, *carnitine palmitoyl transferase-1β* (*CPT-1β)* and *uncoupling protein-3 (UCP-3)* [25]. UCP-3, located in the inner membrane of the mitochondria, is associated with thermogenesis by reducing the proton gradient across the inner mitochondrial membrane [26]. CPT-1β, located in the outer membrane of the mitochondria, is associated with FFA metabolism [27]. Cy3G (10 μM) increased the expression of *CPT-1β* and *UCP-3* by 1.5-fold and 2.7-fold, respectively. It has been reported that UCP-3 expression in skeletal muscle is highly and rapidly induced by swimming, running, and hypoxia compared to other mitochondrial transporters, such as CPT-1, to activate oxidative metabolism [28]. These results indicate that Cy3G can increase metabolic activity in the skeletal muscle by upregulating *PGC-1α*. Muscle-specific overexpression of PGC-1α in mice increased exercise capacity, fatigue resistance, and oxygen uptake [29]. Furthermore, muscle-specific PGC-1α knockout mice showed reduced exercise capacity, muscle function, oxidative metabolism activity, and abnormal glucose homeostasis [30]. Therefore, PGC-1α has been suggested to be an important regulator of exercise adaptation in skeletal muscle and prevent metabolic diseases [30, 31]. In this study, it was hypothesized that Cy3G is effective for enhancing exercise performance and preventing metabolic disorders by regulating metabolic environments. This thesis investigated the effect and regulatory mechanisms of Cy3G on the metabolic environment using animal experiments and *in vitro* cellular model experiments. If the

beneficial health effects and mechanisms of Cy3G can be confirmed, consumption of vegetables and fruits containing Cy3G, such as black soybeans, blueberries, and grapes, may increase, thereby contributing to the development of sustainable agricultural activity.

This Ph.D. thesis consists of 4 chapters. Chapter 1 is a general introduction of the thesis.

Chapter 2 describes the effect of Cy3G on the metabolic activity of skeletal muscle and exercise performance. Regular exercise and physical training contribute to the prevention and alleviation of numerous lifestyle-related diseases, such as obesity, type 2 diabetes, sarcopenia, and cardiovascular disease, as well as enhances physiological capacity in sports by regulating the metabolic environment [32]. It is thought that enhancing the function of skeletal muscle is effective for both the prevention and improvement of metabolic diseases and enhancement of exercise performance [32]. Therefore, the effects and regulation mechanisms of Cy3G on muscle metabolism and exercise were investigated using exercised mice and an *in vitro* model of skeletal muscle cells.

Chapter 3 presents the prevention effect of Cy3G on T2DM as an example of metabolic disease. T2DM patients exhibit an altered metabolic environment such as low energy consumptions and high blood glucose concentration [29, 33, 34]. It is known that adipocytes regulate the metabolic environment (energy balance and glucose homeostasis) through the regulation of metabolic activity and adipokine secretions [33, 34]. Recently, brown-like adipocytes known as beige or brite (brown in white) adipocytes, which appear in the WAT, have gained attention, as beige adipocytes can contribute to the regulation of whole-body energy expenditure [35, 36, 37]. Therefore, an increase in the number or activity level of beige adipocytes in WAT is effective for maintaining good health or preventing T2DM and lifestyle-related diseases [36]. In Chapter 3, the T2DM prevention effect of Cy3G was evaluated using T2DM model mice and an *in vitro* model of adipocytes.

Finally, all results from the above chapters are summarized and perspectives are described in Chapter 4.

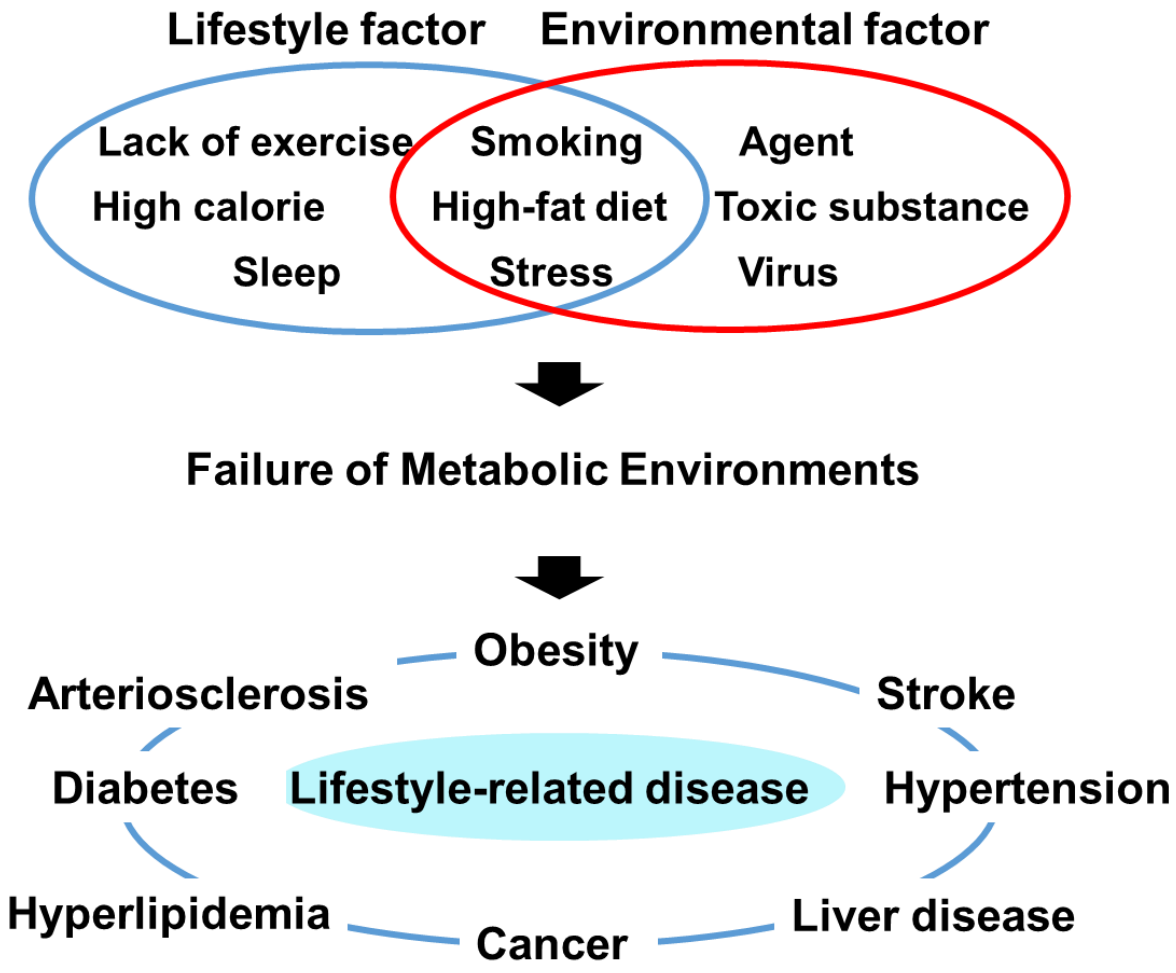


Figure 1-1. Several lifestyle factors and environmental factors involved in the development of lifestyle-related diseases, known as "Seikatsusyukanbyo" in Japan.

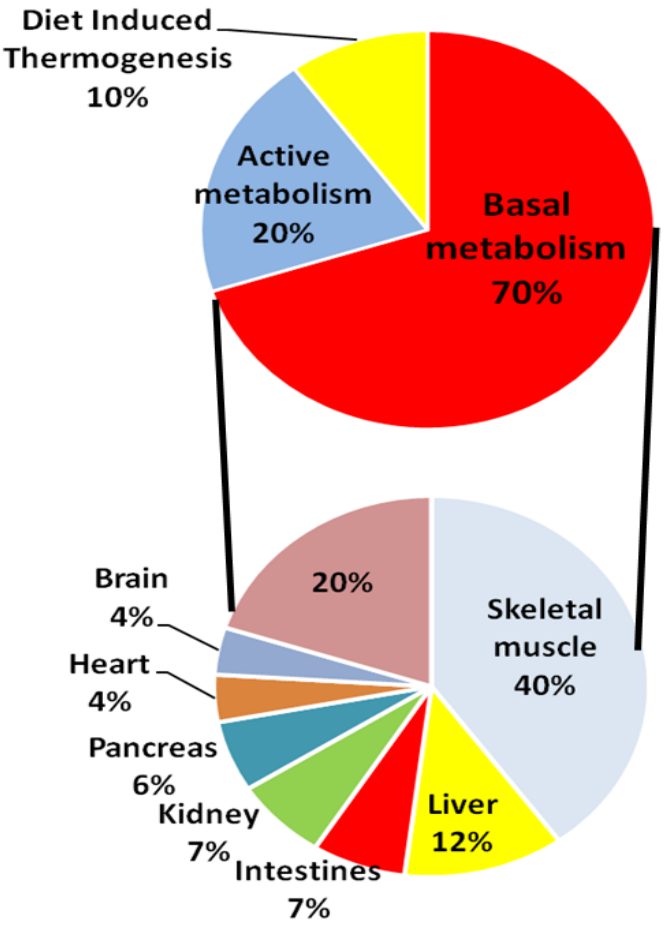


Figure 1-2. Metabolic activity is classified into 3 groups.

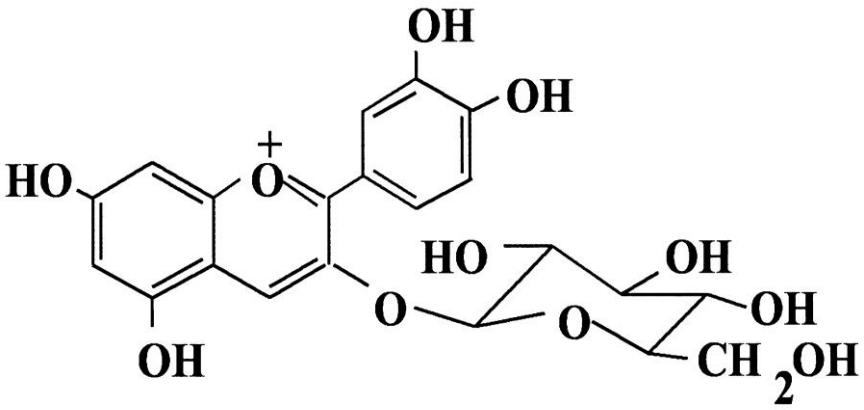


Figure 1-3. Chemical structure of cyanidin-3-glucoside (Cy3G).

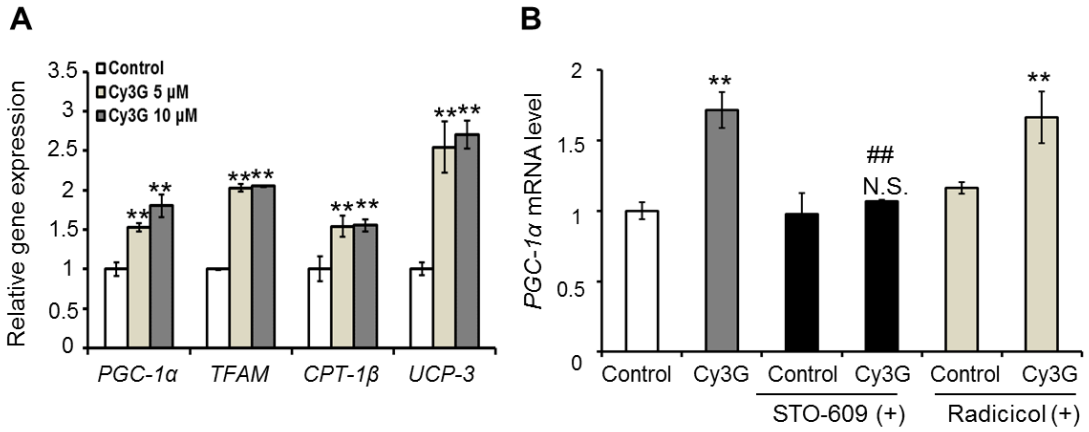


Figure 1-4. Cy3G increased *PGC-1α* upregulation via the calcium/calmodulin-dependent protein kinase kinase (CaMKK) pathway in C2C12 myotubes [23].
C2C12 myotubes were treated with or without Cy3G (5 or 10 μM) for 6 h and then *PGC-1α* mRNA levels were evaluated (A). C2C12 myotubes were pre-treated with or without STO-609 (1 μg/ml) or Radicicol (10 μM) for 30 min. Cy3G (10 μM) treatment was then carried out with or without STO-609 or Radicicol for 6 h and then *PGC-1α* mRNA levels were evaluated. Values were normalised to the *β-actin* expression level and expressed as the mean ± standard deviation of triplicate experiments. ** $P < 0.01$ indicate a significant difference from the control group. ## $P < 0.01$ indicates a significant difference from the Cy3G group. N.S. indicates that the mean value is not significantly different from that of the STO-609-treated control group.

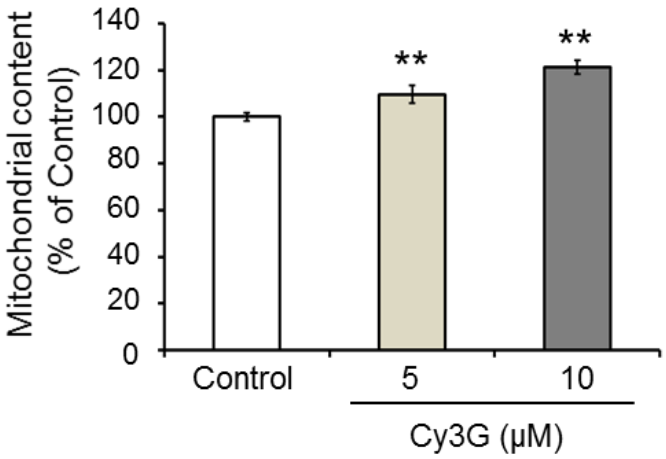


Figure 1-5. Cy3G increased mitochondrial content in C2C12 myotubes [23].
Differentiated C2C12 myotubes were treated with or without Cy3G (5 or 10 μM) for 24 h, after which, the mitochondria content was evaluated. Mitochondria were stained by a mitochondrial specific dye, rhodamine 123. To quantify the rhodamine 123 content, cells were lysed using 1% Triton X-100 and fluorescence intensity (485 nm/528 nm) was measured. Values are expressed as the mean ± standard deviation of triplicate experiments. ** $P < 0.01$ indicate a significant difference from the control group.

Chapter 2

Cyanidin-3-glucoside enhances exercise performance by increasing skeletal muscle PGC-1α expression through the elevation of cAMP levels

Chapter 2. Cyanidin-3-glucoside enhances exercise performance by increasing skeletal muscle PGC-1α expression through the elevation of cAMP levels

The content of this chapter has been published in the following paper with partial modifications:
Matsukawa T, Motojima H, Sato Y, Takahashi S, Villareal OM, Isoda H. Upregulation of skeletal muscle PGC-1α through the elevation of cyclic AMP levels by Cyanidin-3-glucoside enhances exercise performance. Scientific Reports (*under review*)

2.1 Introduction

Enhancing the metabolic rate (increase in energy consumption) in the human body by exercise and activation of metabolic function are effective methods for maintaining the metabolic environment and can prevent lifestyle-related diseases [1, 3, 4]. Skeletal muscle is considered to be the main organ of metabolism since it accounts for 40% of basal metabolism and is involved in physical activity, such as exercise [38, 39]. Elevated muscle metabolism and function also can contribute to exercise performance as well as prevention of lifestyle-related diseases [1, 3, 32]. The reasoning for this is that skeletal muscle is the largest organ in the human body (about 40 % of body mass) and is rich in mitochondria [38, 39]. Mitochondria have a crucial role in energy consuming processes, such as ATP generation and thermogenesis. The decline in mitochondrial number and function with age, lack of exercise, and a sedentary lifestyle are the primary causes of the epidemic of lifestyle-related diseases [5]. It is believed that enhancing the metabolism of skeletal muscle can be effective for both an increase in exercise performance and prevention and/or amelioration of lifestyle-related diseases [32].

Regular exercise and physical training enhances endurance and can prevent or alleviate numerous lifestyle-related diseases, such as obesity, T2DM, sarcopenia, and cardiovascular disease [32]. Physical activity promotes mitochondrial biogenesis and oxidative activity, which has a positive effect on an individual's overall energy and decreases age-related degeneration [40]. The expected lifespan without long-standing illness, is 8–10 years longer in physically active people than in physically inactive people [41]. Therefore, regular exercise and physical activity are effective for enhancing exercise performance as well as human health [32, 40].

PGC-1α is a transcriptional coactivator that plays an important role in the

regulation of metabolism [24]. The expression level of PGC-1α in the skeletal muscle is involved in several adaptations to exercise such as mitochondrial biogenesis, angiogenesis, oxidative metabolism activation, and muscle growth [32, 40, 42]. Muscle-specific overexpression of PGC-1α in mice increases exercise capacity, fatigue resistance, and oxygen uptake [29]. Conversely, muscle-specific PGC-1α knockout mice showed reduced exercise capacity, muscle function, oxidative metabolism activity, and abnormal glucose homeostasis [30]. The downregulation of PGC-1α has been observed in individuals with metabolic diseases, such as obesity and T2DM, and is associated with dysfunction of mitochondrial activity in skeletal muscles [43, 44]. Therefore, it is well established that PGC-1α is known to be an important regulator in exercise adaptation in skeletal muscle and prevention of metabolic disease [30, 31].

Nutraceuticals, which are part of foods, such as dietary fiber, probiotics, vitamins, and polyphenols have recently gained attention as an alternative to pharmaceuticals, since they have shown physiological benefits related to the prevention of diseases [7]. For example, supplementing with the polyphenolic compound quercetin in non-exercising young adult males can enhance exercise performance by inducing mitochondrial biogenesis in skeletal muscles [45]. PGC-1α induces mitochondrial biogenesis through increasing expression of TFAM [24]. In a previous study, it was found that Cy3G increases *PGC-1α* and *TFAM* expression and mitochondrial content in C2C12 myotubes (Fig. 1-4 and 5) [23]. The expression level of PGC-1α in the skeletal muscle depends on several factors, such as mitochondrial biogenesis, angiogenesis, oxidative metabolism activation, and muscle growth [32, 42]. Therefore, Cy3G is predicted to be effective in the activation of metabolic activity of skeletal muscle and the enhancement of exercise performance. However, the mechanism by which Cy3G exerts effects on muscle metabolism and exercise performance has not yet been elucidated. In this chapter, the effects and regulatory mechanisms of Cy3G on muscle metabolism and exercise using cultured skeletal muscle cells and exercised mice are summarized.

2.2 Materials & Methods

2.2.1. Cell culture and differentiation of C2C12 cells

C2C12 myoblasts derived from mouse cell lines were purchased from American Type Culture Collection and cultured in Dulbecco's modified eagle medium (DMEM) (Sigma Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin (5000 μg/ml) – streptomycin (5000 IU/ml) (Lonza, Japan) at 37 ºC in a humidified atmosphere of 5% CO_2 using 75cm^2 flask (Corning, NY, USA). In each experiment, passaging number of 3~7 cells were used. Myoblasts differentiate into myotubes, a widely used *in vitro* model of skeletal muscle, which has the function of muscle contraction and metabolisms [46]. In order to induce differentiation into C2C12 myotubes, C2C12 myoblasts were seeded at 3×10^4 cells/cm^2 and when the approximate confluence were reached (almost 2 days), the medium was changed to DMEM containing 2% horse serum (HS) (Gibco, USA) that was freshly changed every 2 days until full differentiation of C2C12 myotubes (almost 5~6 days).

2.2.2. Cell culture and differentiation of normal human skeletal muscle myoblasts

Normal human skeletal muscle myoblasts were purchased from Lonza, Japan. Cells were cultured in a SkGM-2 Bullet Kit (Lonza, Japan) at 37 °C in a humidified atmosphere of 5% CO_2 using 75cm^2 flask at 4,000 cells/cm^2. Growing to 50~70% confluence of cells was used for the pass-culture. In each experiment, passaging number of 2 or 3 cells were used. To induce the differentiation of Human Skeletal Muscle Myotubes (HSMM), myoblasts were seeded at 4,000 cells/cm^2 and when cultured to 60–70% confluence (almost 2 days), the medium was changed to DMEM:F-12 (Lonza, Japan) containing 2% HS and cultured for 5 days. The growth medium was changed every other day.

2.2.3. Measurement of mitochondrial content using Rhodamine 123

Rhodamine 123 was used for evaluating the number of mitochondria [47]. Rhodamine 123 was purchased from Wako, Japan and dissolved in dimethyl sulfoxide (DMSO) at 100 mg/ml. After treatment, C2C12 myotubes 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. To quantify the rhodamine 123 content, cells were lysed using 1% Triton X-100 (Sigma, USA) and the fluorescence intensity (excitation/emission 485/528 nm) was measured using a Powerscan HT plate reader (Dainippon Sumitomo Pharma Co, Ltd., Japan). The rhodamine 123 content was calculated using the standard curve and values were calculated as a percentage (%) of control.

2.2.4. Transfection of siRNA

C2C12 myoblasts were differentiated into myotubes at 6-well plate as described above. After differentiation, C2C12 myotubes were transfected with *PGC-1α* siRNA (siRNA ID: s72017, Ambion, CA, USA) or *Silencer*® Select Negative Control No. 1 siRNA (Ambion, CA, USA) using Lipofectamine 3000 (Life Technologies, Japan). Briefly, Lipofectamine 3000, 10 μM siRNA solution and Opti MEM Medium (Life Technologies, Japan) were mixed (Lipofectamine 3000: 7.5 μl; 10 μM siRNA: 2.5 μl; Opti-MEM: 125 μl) and after incubation for 5 mins at room temperature. The mix solution (250 μl) was added to cell culture medium (DMEM 10 % FBS: 2250 μl) and incubated for 48 h. After transfection, C2C12 myotubes were used for later experiments. *Silencer*® Select Negative Control No. 1 siRNA was used as negative control.

2.2.5. Measurement of intracellular ATP levels

The intracellular ATP level in C2C12 myotubes were measured using "Cellno" ATP ASSAY reagent (TOYO Ink, Japan) according to the manufacturer's instructions. Briefly, C2C12 myoblasts were seeded at 3×10^4 cells/cm^2 in white, clear-bottom tissue culture 96-well plates (Corning, NY, USA) and were differentiated into C2C12 myotubes as described above. After treatment, ATP assay reagents were added to each well (100 μl/well) and incubated for 10 min at 25 ºC. After that, the luminescence intensity was measured using a Powerscan HT plate reader. The values were calculated as a percentage (%) of control.

2.2.6. Animal experiments

Five-week-old male ICR mice were obtained from Charles River Laboratories (Kanagawa, Japan). The mice were maintained under a 12 h light/dark cycle and had free access to water and a normal diet (MF, Oriental Yeast Co., Ltd., Japan). After 1 week of acclimatisation to laboratory condition, the mice were randomly divided into three groups (n = 7 per group): Group 1 (no exercise group) was orally administered water without swimming exercise. Groups 2 and 3 performed swimming exercise. Group 2 (control group) was orally administered water. Group 3 (Cy3G group) was orally administered 1 mg/kg of Cy3G. Cy3G was purchased from Tokiwa Phytochemical Co. Ltd. (Chiba, Japan) and dissolved in D.W. Animals were orally administrated with 100 μl sample solution using animal feeding needles (Group 1 and 2: D.W. or Group 3: 1 mg/kg Cy3G dissolved in water). Oral administration was performed every day, 1 h before the swimming exercise and at the same time on the no exercise days for 15 days. Body weight was measured daily and food intake was measured every week. The

exercise protocol was adapted from a study by Takeda K et al. with some modifications [48]. Mice were trained to perform the swimming exercise for 10 min with a load corresponding to 5% of their body weight attached to their tails in a tank (30 × 30 × 40 cm) filled with water to a depth of 25 cm, kept at 30 ± 1°C. The swimming exercise was performed every other day for 14 days. On day 15, an exhaustion swimming test was carried out. The mice were made to swim to exhaustion with a load corresponding to 10% of their body weight. Each mouse was considered to reach exhaustion when it failed to raise its face to the water surface within 5 s. Blood lactate levels were measured before and after exercise (0 and 60 min) using Lactate Pro 2 (Arkrey, Japan). Blood glucose levels were measured after exercise (0 min) using Glucose pilot (Iwai Chemicals Company, Japan). Blood samples for lactate and glucose determination were collected from the tail vein. Before being sacrificed, the mice were subjected to fasting for 16 h. After that, blood and tissues (liver, gastrocnemius and biceps femoris) were collected. The serum was collected by centrifugation at 3000 × *g* for 10 min and biochemical parameters (Urea nitrogen, Creatinine, Total ketone bodies, aspartate transaminase, alanine transaminase, alkaline phosphatase and non-esterified fatty acid) of serum were measured by Oriental Yeast Co., Ltd (Japan). All animal experiments were complied with the guidelines of the University of Tsukuba's Regulation of Animal Experiments and were approved by the International Animal Care and Use Committee of the University of Tsukuba (Approved number: 15-069).

2.2.7. Quantification of mRNA levels by Real-Time PCR

Total RNA was isolated from tissue samples (50 mg), C2C12 myotubes and HSMM using ISOGEN (NipponGene, Tokyo, Japan) following the manufacturer's instructions. The RNA pellets were dissolved to Tris EDTA Buffer solution pH 8.0 (Sigma, Japan). The concentration of RNA solution was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcription reactions were carried out with Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) using 2720 Thermal CyCler (Applied Biosystems, CA, USA). 1 μg of total RNA was used for each reverse transcription reactions. 1 μg of RNA solution, 10 mM dNTP (Invitrogen, USA) 1 μl, Oligo (dT) 12-18primer (Invitrogen) 1 μl, and UltraPure DNase/RNase-Free Distilled Water (Gibco, USA) were mixed and incubated at 65 ºC for 5 min. After that, solutions were incubated at on ice for 1 min and added to 5×RT buffer 4 μl, 0.1M DTT 2 μl, Superscript III reverse transcriptase 1 μl, RNase out 1 μl. Subsequently, solutions were incubated at 42 ºC for 60 min and 70 ºC for 10 min. Synthesis of cDNA solution was quantified using a Nanodrop 2000 spectrophotometer and stored at -20 ºC until use.

For the quantification of mRNA levels, TaqMan Real-Time PCR amplification reactions were performed using the Applied Biosystems 7500 Fast Real-Time System (Applied Biosystems, CA, USA). All primers and TaqMan Universal PCR Master mix were obtained from Applied Biosystems. In this chapter, for the quantification of gene expression, the following TaqMan probes were used for muscle tissues and C2C12 myotubes: specific primers for *β-actin* (Mm00607939_s1), *PGC-1α* (Mm01208835_m1), *PPARδ* (Mm00803184_m1), *LDHa* (Mm01612132_g1), *LDHb* (Mm01267402_m1), *MCT1* (Mm01306379_m1), *CPT-1β* (Mm00487191_g1), *VEGFα* (Mm00437306_m1), and *IGF1* (Mm00439560_m1). *β-actin* (Hs01060655_g1), *PGC-1α* (Hs01016719_m1), *TFAM* (Hs00273327_s1), *UCP-3* (Hs01106052_m1), and *CPT-1β* (Hs03046298_s1) were used for HSMM. The mRNA levels of all genes were normalised using *β-actin* as an internal control.

2.2.8. Quantification of protein levels by Western blotting

Total protein was isolated from tissue samples (10 mg) and C2C12 myotubes using RIPA buffer containing a protease inhibitor cocktail (Sigma, Japan). Homogenised sample solutions were collected and freezed in liquid nitrogen for 15 mins. Then, freezing solution was dissolved and centrifuged (4˚C, 10000g, 20 mins). The supernatants were used as protein samples and stored at -80˚C until use. Protein concentrations were quantified by 2D-Quant kit (GE healthcare, Sweden).

Protein samples (15 μg) were used for 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using Power Pac HC (Bio-Rad, USA) under 200 V for 50 min, and then, they were transferred to PVDF membrane (Merck Milllpore, UK) using Power Pac HC under 100 V for 1 h. After blotting, blocking was performed at RT for 1 h using Odyssey Blocking Buffer (LI-COR, Inc., Lincoln, NE, USA). Then, the membrane was incubated with the following antibodies: PGC-1α (1:1000 dilution), CREB (1:1000 dilution), Phospho-CREB (Ser133) (1:1000 dilution), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:200 dilution) and β-Actin (1:5000 dilution), at 4°C overnight, then incubated with secondary antibodies: IRDye 800CW donkey anti-rabbit IgG (1:10000 dilution) or IRDye 680LT goat anti-mouse (1: 20000 dilution) at room temperature for 30 min. The signal was detected using the Odyssey Fc Imaging System. PGC-1α, CREB, and Phospho-CREB (Ser133) antibodies were purchased from Cell Signaling Technology (Hertfordshire, UK). GAPDH antibody (6C5) was purchased from Santa Cruz Biotechnology (CA, USA). β-Actin antibody was obtained from Sigma USA.

2.2.9. Measurement of intracellular Ca^{2+} levels

Intracellular Ca^{2+} levels of C2C12 myotubes were measured using a Calcium Kit II-Fluo 4 (Dojindo, Japan) according to the manufacturer's instructions. C2C12 cells were seeded in black clear bottom 96-well plate (Corning, NY, USA) at 3×10^4 cells/cm^2 and were differentiated into myotube as described above. After differentiation, myotubes were pre-incubated with 100 μl /well of Loading buffer (5% Pluronic F-127, 250 mmol/l Probenecid and 1 μg /μl Fluo 4 AM in Hanks'HEPES Buffer) for 30 min and subsequence treated with or without each concentration of Cy3G for 15-90 min. After incubation, fluorescence intensity (excitation/emission 485/528 nm) was measured using a Powerscan HT plate reader. The fluorescent intensity was calculated as a percentage of control and normalized by the value of 0 min.

2.2.10. Measurement of intracellular cAMP levels

The intracellular cAMP levels in C2C12 myotubes were measured using the cAMP-Glo™ MAX assay (Promega, USA) according to the manufacturer's instructions. Differentiated C2C12 myotubes in white, clear-bottom tissue culture 96-well plates were cultured in serum-free DMEM containing 20 mM $MgCl_2$ with or without phosphodiesterase (PDE) inhibitors (500 μM IBMX and 100 μM Ro20-1724) and with or without Cy3G for 15 min. Luminescence was then measured using a Powerscan HT plate reader. Intracellular cAMP levels were calculated based on a standard curve and values were expressed as a percentage (%) of control. In this assay, PDE inhibitors were used to prevent cAMP hydrolysis during the assay. IBMX and Ro 20-1724 were purchased from Sigma (MO, USA).

2.2.11. Statistical analysis

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

2.3 Results

2.3.1. Cy3G increased *PGC-1α* expression and mitochondria content in HSMM

Skeletal muscle is made up of multinucleated myotubes generated from the fusion of several mononucleated myoblasts [46]. Previously, to investigate the effect of Cy3G on *PGC-1α* expression and mitochondria content in skeletal muscle cells, C2C12 myotubes were used as a cellular model of the skeletal muscle. As shown in Fig. 1-4 and 5, Cy3G significantly increased the expression of *PGC-1α* and the number of mitochondria as shown by increased rhodamine 123 content in C2C12 myotubes. PGC-1α increases the expression of TFAM, which leads to the transcription of mitochondrial genes such as, CPT-1β and UCP-3 [25]. UCP-3 and CPT-1β, which are localized in mitochondrial membrane, and are associated with thermogenesis or FFA metabolism, respectively [26, 27]. The expressions of *UCP-3* and *PGC-1α* were increased by Cy3G (Fig. 1-4 A). To confirm the effect of Cy3G on human cells, primary human skeletal muscle myotubes must be used as a model system. As shown in Fig. 2-1 A, Cy3G increased the expression of *PGC-1α*, *TFAM*, *CPT-1β*, and *UCP-3* in HSMM. Furthermore, the number of mitochondria in HSMM was also increased by Cy3G (Fig. 2-1 B).

2.3.2. Cy3G-induced *PGC-1α* upregulation increased mitochondrial content in skeletal muscle cells

To confirm whether Cy3G-induced PGC-1α upregulation is required in mitochondrial biogenesis, the expression of *PGC-1α* in C2C12 myotubes was suppressed by *PGC-1α* siRNA (Fig. 2-2 A). As shown in Fig. 2-2 B, the observed Cy3G-induced elevation of rhodamine 123 content was suppressed by *PGC-1α* knockdown. These results indicate that mitochondrial content in skeletal muscle cells is increased by Cy3G-induced *PGC-1α* upregulation.

2.3.3. Cy3G enhanced lactate metabolism of skeletal muscle cells

Blood lactate levels during exercise are associated with the lactate production to clearance ratio [49, 50]. PGC-1α in the skeletal muscle promotes lactate metabolism by promoting expression of lactate dehydrogenase (LDH) B and monocarboxylate transporter 1 (MCT1) and decreasing LDH A expression (Fig. 2-3) [51]. LDH B catalyses the conversion of lactate to pyruvate and MCT1 facilitates lactate uptake [51]. Conversely, LDH A catalyses the conversion of pyruvate to lactate. To investigate the effect of Cy3G on the expression of involved in lactate metabolism-related genes in skeletal muscle cells, C2C12 myotubes were treated with Cy3G for 24 h. As shown in Fig. 2-4 A, Cy3G (10 μM) increased *LDH B* and *MCT1* expressions in C2C12 myotubes by 1.4-

and 1.5-fold, respectively. However, the expression level of LDH A was not changed (Fig. 2-4 A). In humans, blood lactate levels are elevated to around 20 mM after vigorous exercise [52, 53]. To evaluate the effect of Cy3G on lactate metabolism ability in skeletal muscle cells, Cy3G-treated C2C12 myotubes were cultured in serum- and glucose-free DMEM containing 20 mM lactate for 15 min, and intracellular ATP production was measured. As shown in Fig. 2-6 B, the intracellular ATP production in Cy3G-treated C2C12 myotubes was increased compared to that in the lactate-treated control (87% vs 104%).

2.3.4. Cy3G administration increased swimming time and inhibited exercise-induced lactate elevation

To examine whether Cy3G can enhance exercise performance (swim until exhaustion with a load corresponding to 10% of their body weight), mice were given Cy3G or water (control) orally for 15 days and were made to perform exercise (Fig. 2-5). During the experimental period, animals in three experimental groups consumed the same amount of food (Table 2-1). Blood lactate levels during exercise are associated with the ratio of lactate production to clearance [49, 50]. Lactate accumulation resulting in the production of H^+ ions, in the muscles causes fatigue by depressing muscle function and performance [49]. Blood lactate levels were measured on Day 13 (10 min swimming) and Day 15 (swimming until exhaustion) before and after the swimming exercise. Before exercise, blood lactate levels were not statistically different between the control and Cy3G groups (Fig. 2-6 A and C). However, after exercise, lactate elevation was significantly lower in the Cy3G group on Day 13 (10.7 mM vs 7.2 mM) and Day 15 (12.6 mM vs 8.0 mM) compared to that in the control group (Fig. 2-3 A and C). Additionally, blood glucose levels after exercise in the Cy3G group was maintained at higher levels on Day 13 (136 mg/dL vs 181 mg/dl) and Day 15 (152 mg/dL vs 178 mg/dl) compared to the control group (Fig. 2-6 B and D). Furthermore, as shown in Fig. 2-3 E, time until exhaustion in swimming was increased in Cy3G-administered mice compared to that in the control group (230 seconds in the control group vs 377 seconds in the Cy3G group). The blood levels of urea nitrogen, creatinine, and total ketone bodies were increased after exercise and the accumulation of these biochemical components has been considered as fatigue markers [54]. Cy3G group had lower levels of these biochemical components compared to control (Table 2-2). Moreover, the weight of the gastrocnemius and biceps femoris were increased in Cy3G-administrated mice (Table 2-1).

2.3.5. Cy3G increased PGC-1α expression and regulated lactate metabolism-related genes expression in the gastrocnemius and biceps femoris muscles

Lactate produced by working muscle is released into the blood and taken up by muscle tissue as energetic fuel [50, 52]. To explore the effect of Cy3G on muscles, the gastrocnemius and biceps femoris muscle, which are extensively used during swimming, were isolated [48]. As shown in Fig. 2-7 A and B, in both muscles, Cy3G increased PGC-1α protein levels by 2.6- and 2.7-fold, respectively, compared to the control (exercise + water) group. The gene expression of *PGC-1α* was also increased by 3.3- and 2.4-fold, respectively, compared to that in the control (exercise + water) group (Fig. 2-8 A and B). Additionally, the Cy3G group had increased expression of *LDH B* and *MCT1* in gastrocnemius (Fig. 2-8 A, 1.9- and 1.3-fold, respectively) and biceps femoris (Fig. 2-8 B, 2.5- and 1.3-fold, respectively) compared to the control (exercise + water) group. Furthermore, Cy3G decreased *LDH A* expression in both muscles (0.6- and 0.5-fold, respectively) compared to the control (exercise + water) group (Fig. 2-5 A and B). These results suggest that lactate metabolism in the muscles is enhanced in response to Cy3G-induced PGC-1α upregulation.

2.3.6. Cy3G increased angiogenesis, muscle growth, and lipid metabolism-related genes expression in the gastrocnemius and biceps femoris muscles

To adapt to exercise, the expression level of PGC-1α in the skeletal muscle is upregulated to induce changes in angiogenesis, muscle growth, lipid metabolism, and lactate metabolism [32, 42]. Vascular endothelial growth factor α (VEGFα) and insulin-like growth factor 1 (IGF1), cytokines released from skeletal muscle, important factors angiogenesis and muscle growth, respectively, are regulated by PGC-1α [32, 42]. As shown in Fig. 2-9, Cy3G-treated mice showed increased expression of *VEGFα* and *IGF1* in gastrocnemius (1.6- and 1.2-fold, respectively) and biceps femoris (1.4- and 1.4-fold, respectively) compared to the control group. Additionally, peroxisome proliferator-activated receptor-δ (PPARδ) and CPT-1β are known to be involved in lipid metabolism [55]. As shown in Fig. 2-10, Cy3G-treated mice showed increased expression of *PPARδ* in gastrocnemius (1.7-fold) and biceps femoris (1.6-fold) compared to control group. However, the upregulation of *CPT-1β* was only observed in biceps femoris (1.5-fold). Notably, these changes were weak compared to those of LDH A (0.6- and 0.5-fold) and LDH B (1.9- and 2.5-fold).

2.3.7. Cy3G increased intracellular Ca^{2+} by elevation of intracellular cAMP levels

The activation of AMP-activated protein kinase (AMPK) pathway promotes

mitochondrial biogenesis through PGC-1α [56]. CaMKK and liver kinase B1 (LKB1) are known as kinases upstream of AMPK, which induce AMPK phosphorylation [57]. As shown in Fig. 1-4, STO-609, a CaMKK inhibitor [58], blocked Cy3G-induced *PGC-1α* up-regulation [23]. This result indicates that Cy3G increases *PGC-1α* expression via CaMKK signaling, but not LKB1 signaling, in C2C12 myotubes [23]. CaMKK is activated by the elevation of intracellular Ca^{2+} levels and phosphorylates AMPK through activation of calmodulin kinase (CaMK) [59]. As expected, Cy3G was found to increase intracellular Ca^{2+} (Fig. 2-8 A). Elevated intracellular cAMP levels induce the release of Ca^{2+} from the sarcoplasmic reticulum [60]. As shown in Fig. 2-8 B, Cy3G increases the intracellular cAMP level in C2C12 myotubes to 134%. This elevation was abrogated in the absence of PDE inhibitors. Furthermore, Cy3G slightly increased the phosphorylation of cAMP response element binding protein (CREB) (Fig. 2-12).

2.4 Discussion

The major finding of this chapter is that Cy3G enhances swimming time through PGC-1α upregulation. PGC-1α is a transcriptional coactivator that has an important role in the regulation of adaptation to exercise through mechanisms such as regulating lactate and FFA metabolism, angiogenesis, and mitochondrial biogenesis [32, 42]. In individuals suffering from metabolic diseases, such as obesity, diabetes, and cardiomyopathy, the expression of PGC-1α has been found to be decreased [24]. This chapter summarizes results suggesting that the increase of PGC-1α expression induced by Cy3G contributes to the enhancement of exercise performance as well as the management of lifestyle-related diseases.

Lactate is produced when glucose or glycogen is used as a fuel source. Blood lactate levels during exercise are regulated by lactate production and clearance [49]. Accumulated lactate in the muscles causes fatigue since lactate produces H^+ ions, which depresses muscle function and performance [49]. Furthermore, hypoglycemia, having decreased blood glucose levels, during exercise can also cause fatigue, leading to exercise cessation [61]. Skeletal and cardiac muscle can eliminate circulating lactate by using it as a fuel source [49]. As shown in Fig. 2-6, exercise-induced elevation of lactate levels in Cy3G-treated mice was lower than the increase observed in control mice. The increase of lactate oxidizing phenotypes is associated with the induction of PGC-1α expression in skeletal muscle since PGC-1α increases LDH B and MCT1 expression and decreases LDH A expression [51]. It has been reported that PGC-1α mRNA levels return to "rest level" within a few hours after exercise, while the increase of PGC-1α protein levels is maintained for 18-48 h post exercise [62, 63]. Although, the gastrocnemius and biceps femoris muscles were collected 24 h after the swimming until exhaustion test, this study did not find an increase in mRNA and protein PGC-1α expression in muscles. However, in the Cy3G-treated group, an increase in PGC-1α mRNA and protein expression was observed. Furthermore, Cy3G also increased *LDH B* and *MCT1* expression (Fig. 2-7 and -8). Therefore, these results suggest that increased lactate metabolism by PGC-1α upregulation was due to Cy3G. Exercise training promotes lactate oxidising phenotypes due to the induction of PGC-1α expression in skeletal muscle [51]. Thus, it is likely that both Cy3G and exercise can synergistically activate PGC-1α expression and lactate oxidising phenotypes. To establish the validity of this hypothesis, future investigation is required using exercise protocol, which can induce PGC-1α expression.

MCT1 plays an important role in the transportation of lactate and ketone bodies (β-hydroxybutyrate, acetoacetate, and acetone) [64]. Elevation of PGC-1α in skeletal

muscle increases MCT1 expression and has a central role in ketone body homeostasis [61]. It has been predicted that increasing of *MCT1* expression with Cy3G treatment can decrease total ketone body levels in serum. Supporting this idea, Cy3G was found to reduce serum non-esterified fatty acid (NEFA) levels (Table 2-2). Ketone bodies and FFAs are metabolized by β-oxidation in the skeletal muscle. It has been reported that overexpression of PGC-1α activates the FFA oxidative capacity of skeletal muscle cells by increasing the expression of genes involved in lipid metabolism, such as *CPT-1β* [65]. PPARδ is the most abundantly expressed of the PPAR family in skeletal muscle and is known as a regulator of lipid metabolism [55]. PPARδ also regulates lipid metabolism by increasing expression of the target genes such as CPT-1β and UCP-3 [55]. The decrease of ketone bodies and NEFA levels in Cy3G-treated groups was likely because of PGC-1α and PPARδ upregulation.

Increased mitochondria number and function in skeletal muscle is an important as adaptation to exercise since mitochondria is the critical organelle for ATP generation and thermogenesis [40, 42]. Mitochondrial content of skeletal muscle cells increased in response to Cy3G-induced *PGC-1α* upregulation (Fig. 2-2). PGC-1α interacts with estrogen-related receptor α (ERRα) to induce the transcription of genes related to mitochondrial biogenesis, such as TFAM [66]. Furthermore, PGC-1α and ERRα are also involved in the regulation of lactate metabolism [51]. Therefore, it is likely that *PGC-1α* upregulation by Cy3G promotes mitochondrial biogenesis through interaction with ERRα. It has been reported that the regulation of angiogenesis is also associated with interaction of PGC-1α with ERRα [67]. Muscle vascularization by VEGFα increases muscle blood supply and oxygen availability, thus increasing exercise performance and endurance [68]. This suggests that the increase in *VEGFα* expression in muscle by Cy3G partially enhances mouse performance during exercise.

The AMPK pathway plays an important role in the regulation of cellular metabolism, promoting mitochondrial biogenesis through PGC-1α upregulation [56]. CaMKK is one of the upstream kinases in the AMPK pathway, and is activated in response to elevation of intracellular Ca^{2+} levels [69]. The CaMKK signaling pathway is also involved in the stable induction of PGC-1α by autofeedback regulation [70]. Cy3G was found to increase *PGC-1α* expression via the CaMKK-AMPK pathway in skeletal muscle cells [23]. However, another study found that intracellular cAMP levels in HepG2 hepatocytes are increased with Cy3G treatment [71]. As shown in Fig. 2-8, Cy3G caused the elevation in both intracellular Ca^{2+} and cAMP levels in C2C12 myotubes. Epac1 is one of the cAMP-regulated guanine nucleotide exchange factors, and it activates the release of Ca^{2+} from the endoplasmic reticulum/ sarcoplasmic

reticulum [72]. As shown in Fig. 2-11, after 30 mins after Cy3G treatment, an increase in Ca^{2+} level was detected. Therefore, it is predicted that Cy3G increased intracellular Ca^{2+} levels by elevating intracellular cAMP levels. Therefore, it is predicted that PGC-1α upregulation in response to Cy3G is regulated by Ca^{2+}-CaMKK pathway. It has been reported that the elevation of intracellular cAMP levels induces phosphorylation of CREB which, in turn, can induce PGC-1α expression [73]. As shown in Fig. 2-12, Cy3G slightly increased the phosphorylation of CREB. Future investigation is required, but it is predicted that PGC-1α upregulation in response to Cy3G is regulated by the Ca^{2+}-CaMKK and CREB pathways through the increase of intracellular cAMP levels.

PDE is family enzymes hydrolyze cAMP to 5′AMP to regulate intracellular cAMP levels [74]. In cAMP assays, PDE inhibitors are used to prevent cAMP hydrolysis during the assay. Cy3G treatment increased intracellular cAMP levels in the presence of PDE inhibitors, but did not increase cAMP levels in the absence of PDE inhibitors (Fig. 2-11 B). Dallas *et. al*. used an *in vitro* cell-free assay to demonstrate an inhibitory effect of Cy3G on PDE enzymatic activity [75]. Therefore, it can be concluded that Cy3G increases intracellular cAMP levels through inhibition of PDE activity.

Enhancement of metabolism is effective for improvement of metabolic diseases, such as T2DM and obesity [32]. In metabolic disease patients, a decrease of PGC-1α expression and mitochondrial function in skeletal muscle caused by an increase in inflammatory adipokines and FFA has been observed [76]. In this chapter, the effect of Cy3G treatment on increases in mitochondrial content and PGC-1α expression are examined. Further study is needed, but it is predicted that Cy3G may be an effective therapeutic agent for the prevention and treatment of metabolic diseases. This point is further discussed in Chapter 3. Furthermore, in the regulation of whole-body energy expenditure, brown-like adipocytes called as beige or brite (brown in white) adipocytes have attracted attention [36]. Although their origin is the same as white adipocytes, beige adipocytes have characteristics of brown adipocytes, such as UCP-1 expression, thermogenesis, and higher numbers of mitochondria compared to white adipocytes [35, 36]. The increase in the number or activity of beige adipocytes in WAT is one target to maintain good health and prevent the development of metabolic diseases, since beige adipocytes can contribute to the regulation of whole-body energy expenditure [36]. External stimulation such as exercise, chronic cold exposure, norepinephrine, irisin, PPARγ agonists, and cAMP can induce formation of beige adipocytes [77]. As shown in Fig. 2-11, intracellular cAMP levels were elevated in response to Cy3G treatment. Therefore, it is predicted that Cy3G may be able to induce the preadipocyte differentiation

into beige adipocytes. This point is also further discussed in Chapter 3.

In summary, this chapter highlights that increasing lactate metabolism in response to Cy3G-induced PGC-1α upregulation enhanced swimming time and reduced fatigue. Cy3G-induced *PGC-1α* upregulation also increased mitochondrial content in the muscle cells. Moreover, the elevation of intracellular cAMP levels by Cy3G induced *PGC-1α* upregulation via CaMKK and CREB pathways (Fig. 2-12).

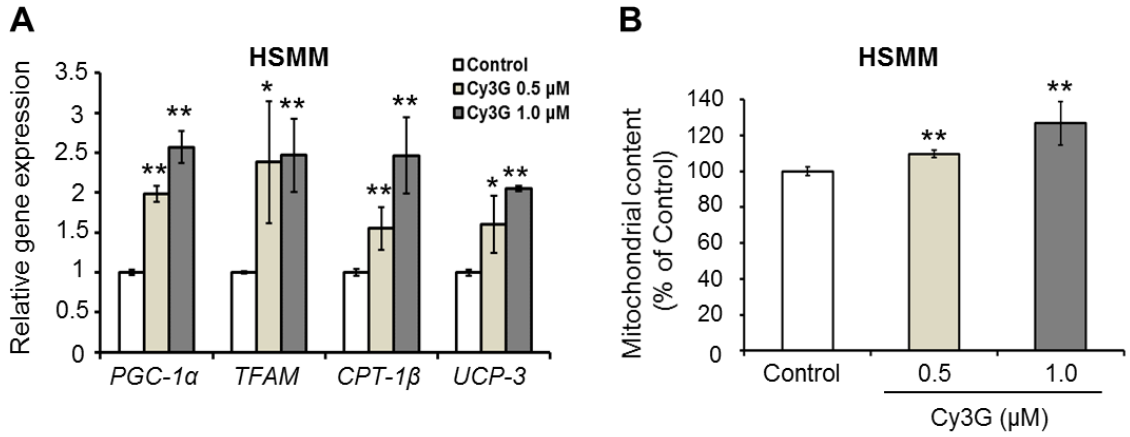


Figure 2-1. Effect of Cy3G on the expression of *PGC-1α* and mitochondrial content in HSMM.

Differentiated HSMM were treated with or without Cy3G for 6 h (A) or 24 h (B), after which, the gene expression of *PGC-1α*, *TFAM*, *CPT-1β*, and *UCP-3* (A) and the mitochondria content (B) were evaluated. (A) Expression levels of mRNA were normalised to the *β-actin* expression level and expressed relative to the control. Values are expressed as the mean ± standard deviation of triplicate experiments. * $P < 0.05$ and ** $P < 0.01$ indicate a significant difference from the control group.

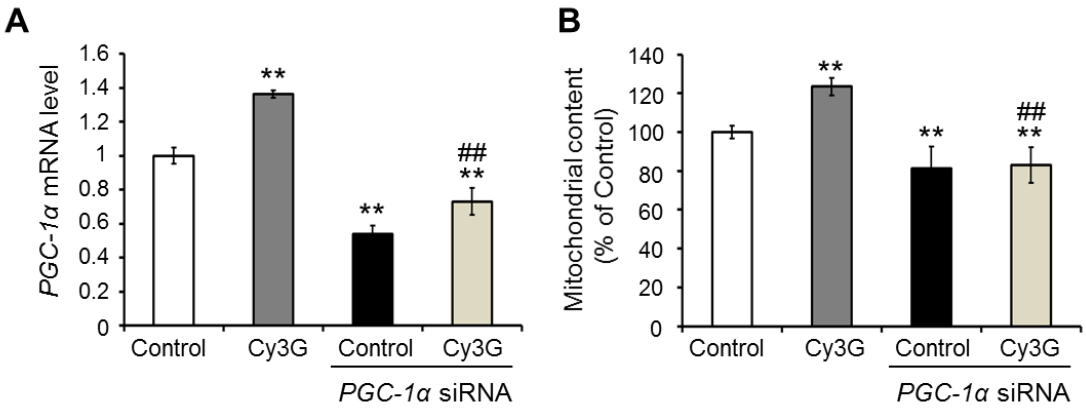


Figure 2-2. Effect of Cy3G-induced *PGC-1α* upregulation on mitochondrial content in C2C12 myotubes.

Differentiated C2C12 myotubes were transfected with *PGC-1α* siRNA or Control siRNA for 48 h and then, treated with or without Cy3G for (A) 6 h or (B) 24 h. Following treatment, *PGC-1α* mRNA levels (A) and mitochondria content (B) were evaluated. (A) Expression levels of mRNA were normalised to the *β-actin* expression level and expressed relative to the control. Values are expressed as the mean ± standard deviation of triplicate experiments. * $P < 0.05$ and ** $P < 0.01$ indicate a significant difference from the control group. ## $P < 0.01$ indicates a significant difference from the Cy3G group.

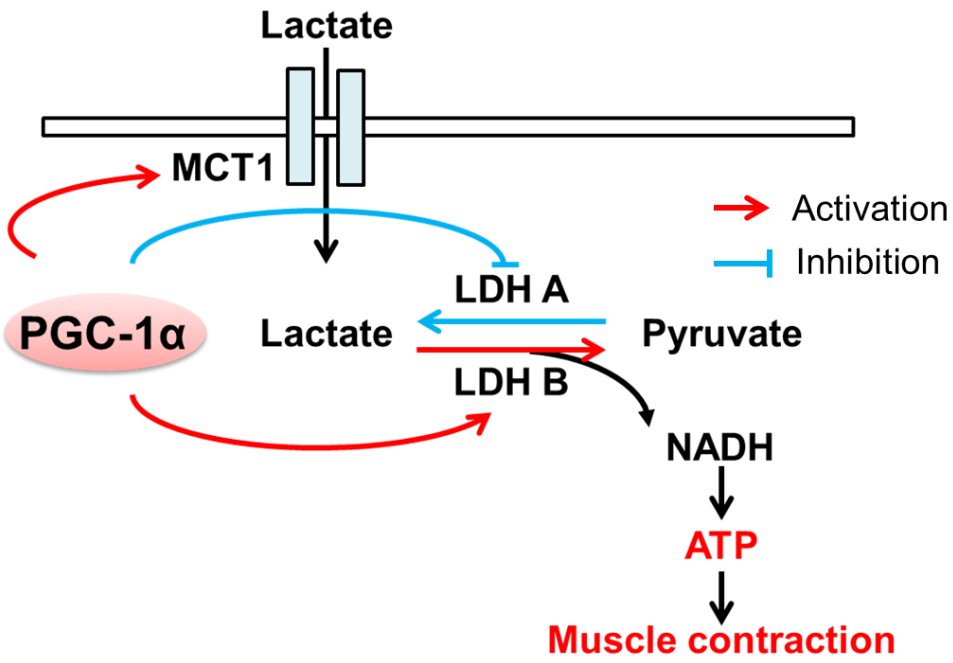


Figure 2-3. Schematic diagram of the regulation of lactate metabolism by PGC-1α. PGC-1α is the transcriptional coactivator of several genes such as glucose uptake, mitochondrial biogenesis, lactate metabolism and angiogenesis. PGC-1α in the skeletal muscle regulates blood lactate levels by increasing lactate dehydrogenase (LDH) B and monocarboxylate transporter 1 (MCT1) and decreasing LDH A expression. LDH B catalyses the conversion of lactate to pyruvate and MCT1 facilitates lactate uptake. While, LDH A catalyses the conversion of pyruvate to lactate [51].

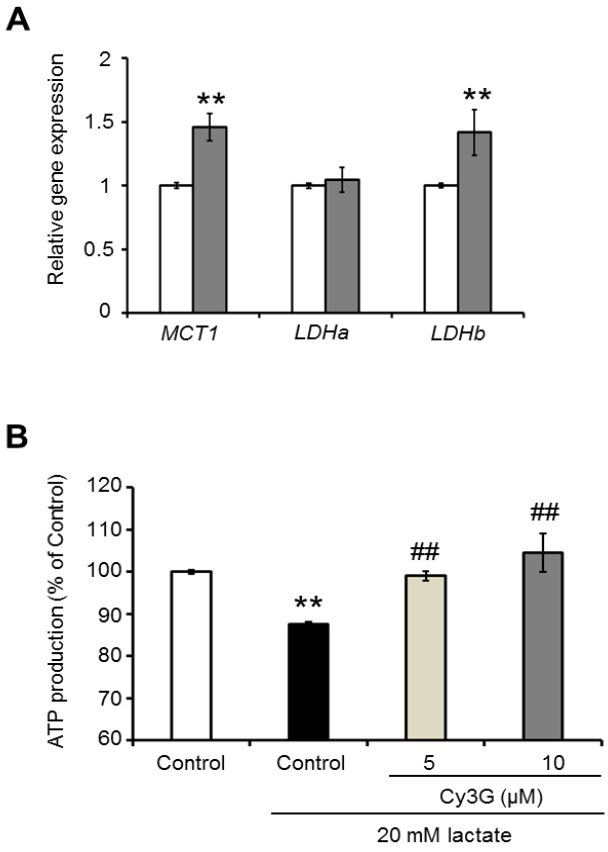


Figure 2-4. Effect of Cy3G on lactate metabolism of C2C12 myotubes.
Differentiated C2C12 myotubes were treated with or without Cy3G for 24 h. After that, mRNA expression levels of *MCT1*, *LDHa*, and *LDHb* were evaluated. Gene expression levels were normalised to the *β-actin* expression level (A). After Cy3G treatment (24 h), C2C12 myotubes were cultured with serum- and glucose-free DMEM containing 20 mM lactate for 15 min and then, intracellular ATP production in C2C12 myotubes were evaluated (B). Values are expressed as the mean ± standard deviation of triplicate experiments. ** $P < 0.01$ indicates a significant difference from the control group. $^{\#}$ $P < 0.05$ and $^{\#\#}$ $P < 0.01$ indicate a significant difference from the lactate-treated control.

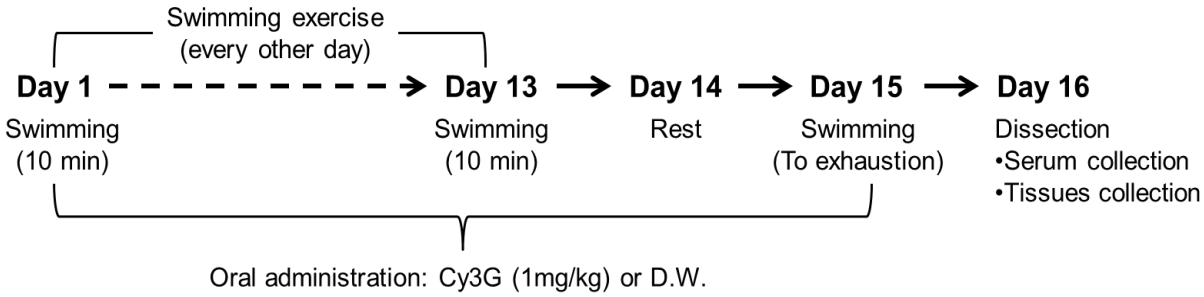


Figure 2-5. Experimental schedule of animal experiments.

Mice were trained to perform a swimming exercise and performed the exercise every other day for 14 days, and an exhaustion swimming test was carried out on day 15.

Table 2-1. Body weight, food intake and the weight of liver, gastrocnemius and biceps femoris in mice.

		Swimming	
	No exercise	Water	Cy3G
Initial body weight (g)	28.7 ± 1.3	28.2 ± 1.4	28.5 ± 0.8
Final body weight (g)	30.7 ± 1.2	31.6 ± 2.0	32.5 ± 1.7
Food intake (g/day)	3.67 ± 0.12	3.67 ± 0.22	3.79 ± 0.16
Liver (g)	1.19 ± 0.04	1.18 ± 0.04	$1.29 \pm 0.04^{**, \#\#}$
Gastrocnemius (g)	0.22 ± 0.03	0.23 ± 0.02	$0.29 \pm 0.04^{*, \#}$
Biceps femoris (g)	0.26 ± 0.03	$0.34 \pm 0.0^{**}$	$0.56 \pm 0.02^{**, \#\#}$

Values are expressed as the mean ± standard deviation. * $P < 0.05$ and ** $P < 0.01$ indicate a significant difference from the no exercise group. $^{\#}$ $P < 0.05$ and $^{\#\#}$ $P < 0.01$ indicate a significant difference from the exercise + water group.

Table 2-2. The level of serum biochemical parameters in mice.

		Swimming	
	No exercise	Water	Cy3G
Urea nitrogen (mg/dL)	32.3 ± 0.9	28 ± 3.5	22 ± 1.8$^{\#}$
Creatinine (mg/dL)	0.15 ± 0.01	0.20 ± 0.05	0.12 ± 0.02$^{\#}$
Total ketone bodies (μM)	593.3 ± 53.3	1282.0 ± 210.4**	847.8 ± 143.5$^{*, \#}$
AST (IU/L)	1690.0 ± 258.9	2082.5 ± 334.3	2075.0 ± 388.9
ALT (IU/L)	226.3 ± 3.5	226.8 ± 40.4	160.2 ± 103.0
ALP (IU/L)	345.0 ± 17.9	433.7 ± 37.6**	347.5 ± 40.9$^{\#}$
NEFA (μEq/L)	1125.8 ± 140.9	1298.2 ± 129.8	1080.0 ± 122.6$^{\#}$

Values are expressed as the mean ± standard deviation. * $P < 0.05$ and ** $P < 0.01$ indicate a significant difference from the no exercise group. $^{\#}$ $P < 0.05$ indicates a significant difference from the exercise + water group. AST: aspartate transaminase, ALT: alanine transaminase, ALP: alkaline phosphatase, NEFA: non-esterified fatty acid.

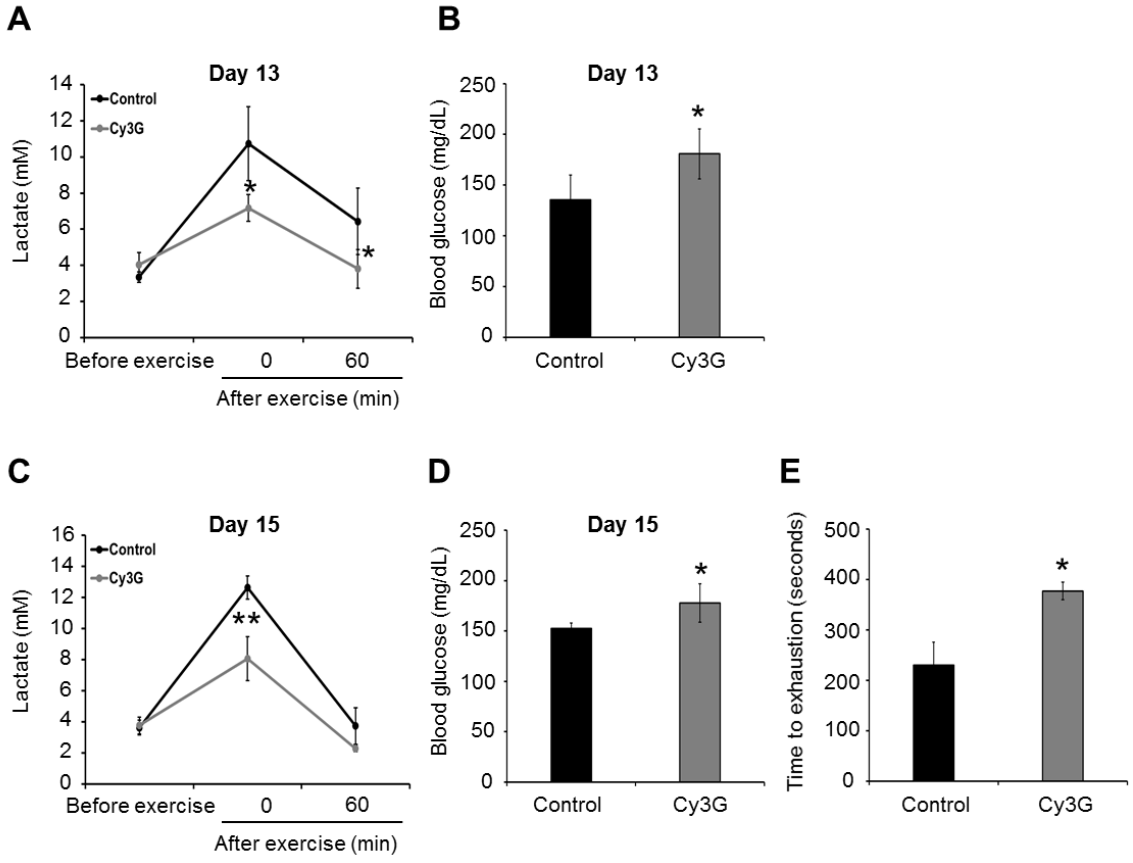


Figure 2-6. Effect of Cy3G on blood lactate and glucose levels.

Mice were trained to perform a swimming exercise and performed the exercise every other day for 14 days, and an exhaustion swimming test was carried out on day 15. On days 13 and 15, (A, B) blood lactate levels before and after exercise (0 and 60 min) and (C, D) blood glucose levels after exercise (0 min) were evaluated. (E) On day 15, swimming time to exhaustion was measured. Values are expressed as the mean ± standard deviation. * $P < 0.05$ and ** $P<0.01$ indicate a significant difference from the control (water) group.

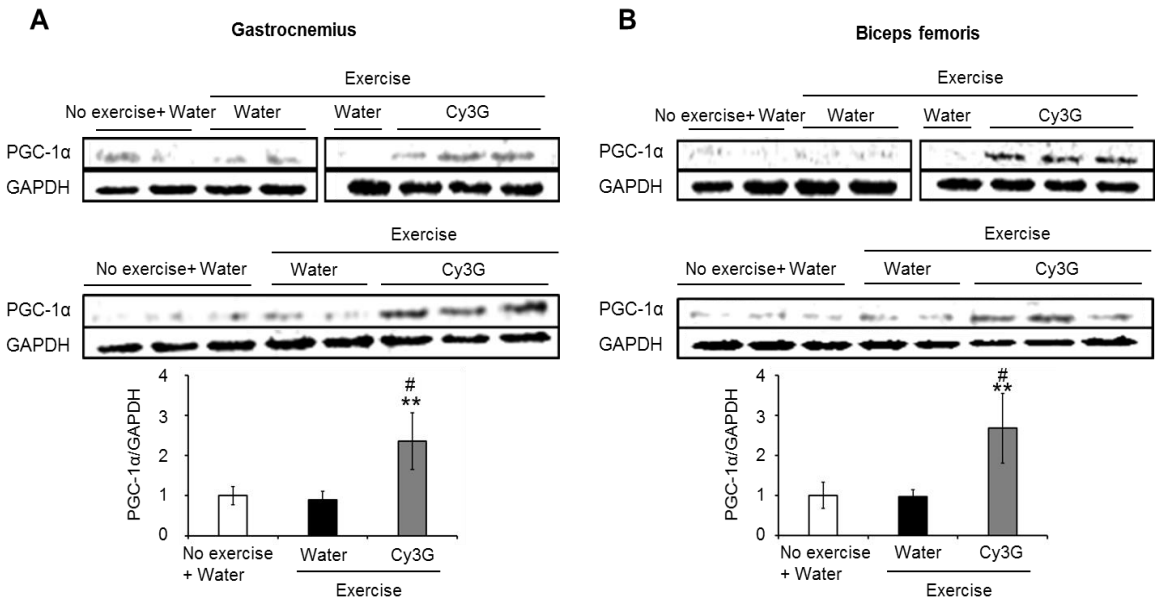


Figure 2-7. Effect of Cy3G on protein expression of PGC-1α in the gastrocnemius and biceps femoris.

Protein expression levels of PGC-1α in the gastrocnemius (A) and biceps femoris (B) were evaluated. (A, B) Protein expression levels were normalised to the expression of GAPDH. All gels were run under the same experimental conditions and the representative blots were shown. Values are expressed as the mean ± standard deviation and relative to the no exercise group. ** $P < 0.01$ indicate a significant difference from the no exercise group. # $P < 0.05$ indicate a significant difference from the exercise + water group.

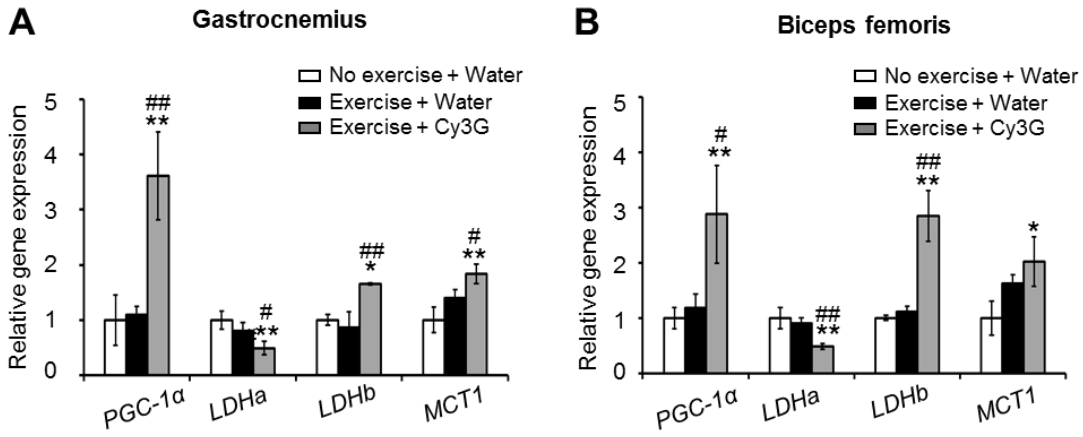


Figure 2-8. Effect of Cy3G on the expression of lactate metabolism-related genes in the gastrocnemius and biceps femoris.

Expression levels of PGC-1α and lactate metabolism-targeted genes in the gastrocnemius (A) and biceps femoris (B) were evaluated. (A, B) Expression levels of mRNA were normalised to the *β-actin* expression level. Values are expressed as the mean ± standard deviation and relative to the no exercise group. * $P < 0.05$ and ** $P < 0.01$ indicate a significant difference from the no exercise group. $^{\#}$ $P < 0.05$ and $^{\#\#}$ $P < 0.01$ indicate a significant difference from the exercise + water group.

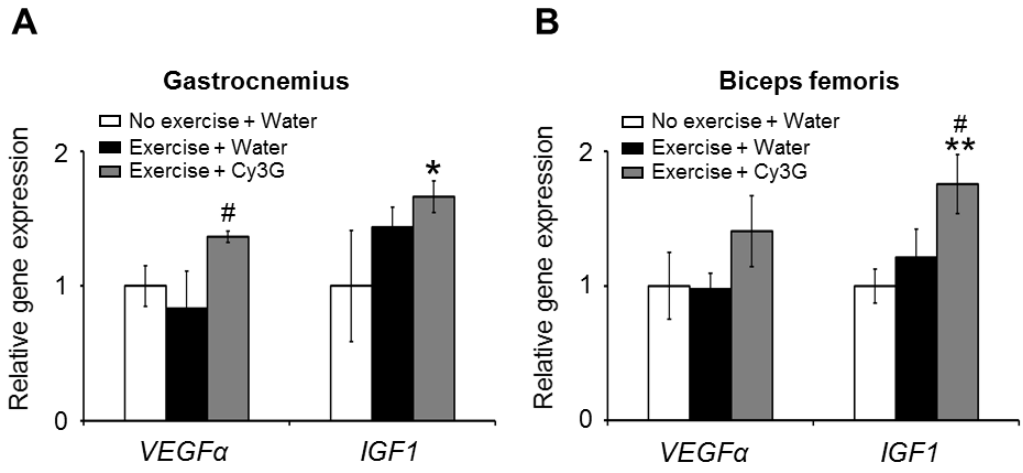


Figure 2-9. Effect of Cy3G on the expression of *VEGFα* and *IGF1* in the gastrocnemius and biceps femoris.

Expression levels of *VEGFα* and *IGF1* in the gastrocnemius (A) and biceps femoris (B) were evaluated. (A, B) Expression levels of mRNA were normalised to the *β-actin* expression level. Values are expressed as the mean ± standard deviation and relative to the no exercise group. * $P < 0.05$ and ** $P < 0.01$ indicate a significant difference from the no exercise group. $^{\#}$ $P < 0.05$ indicate a significant difference from the exercise + water group.

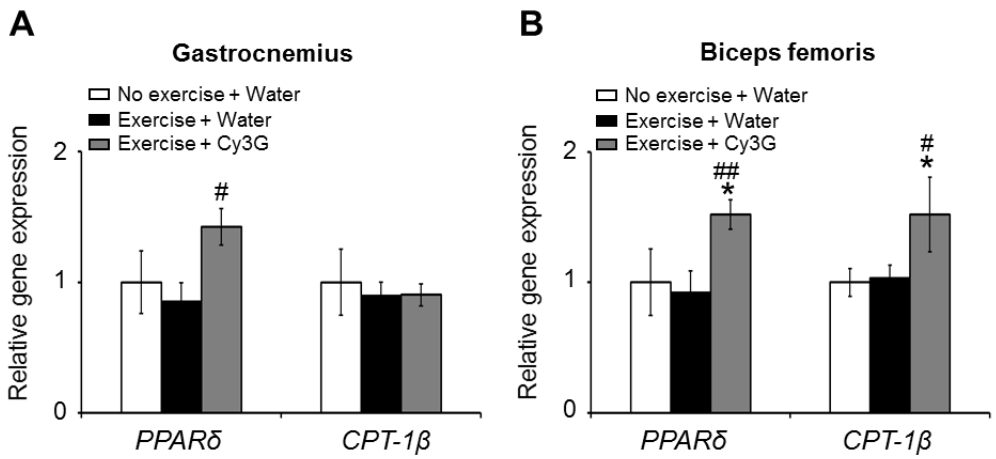


Figure 2-10. Effect of Cy3G on the expression of lipid metabolism-related genes expression in the gastrocnemius and biceps femoris.

Expression levels of *PPARδ* and *CPT-1β* in the gastrocnemius (A) and biceps femoris (B) were evaluated. (A, B) Expression levels of mRNA were normalised to the *β-actin* expression level. Values are expressed as the mean ± standard deviation and relative to the no exercise group. * $P < 0.05$ and ** $P < 0.01$ indicate a significant difference from the no exercise group. $^{\#}$ $P < 0.05$ indicate a significant difference from the exercise + water group.

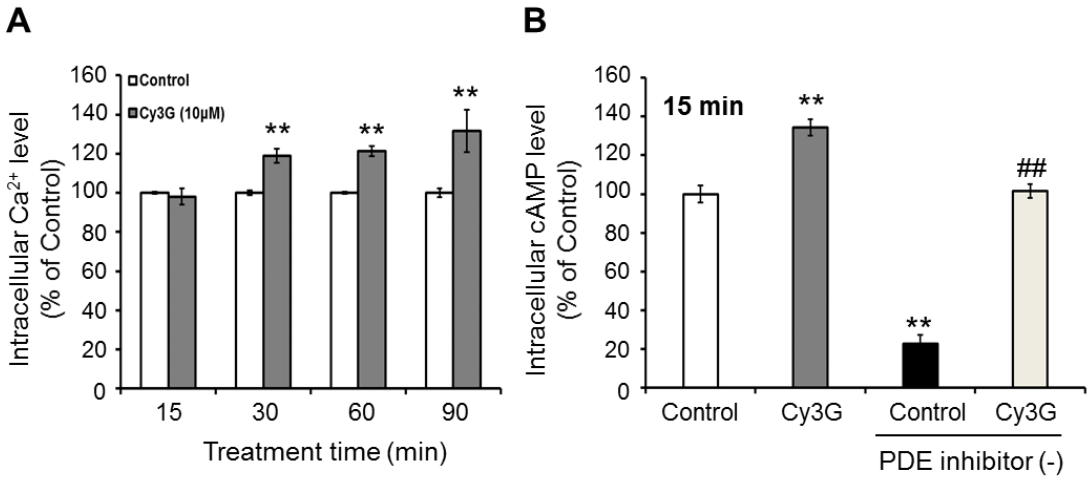


Figure 2-11. Effect of Cy3G on intracellular Ca^{2+} and cAMP levels in C2C12 myotubes.

To evaluate the effect of Cy3G on intracellular Ca^{2+} levels, C2C12 myotubes were pre-incubated with Fluo4 AM for 30 min and subsequently treated with or without Cy3G (10 μM) for 15–90 min (A). To evaluate the effect of Cy3G on intracellular cAMP levels, C2C12 myotubes were treated with or without Cy3G (10 μM) and with or without PDE inhibitors (500 μM IBMX and 100 μM Ro20-1724) for 15 min (B). Values are expressed as the mean ± standard deviation of triplicate experiments. * $P < 0.05$ and ** $P < 0.01$ indicate a significant difference from the control group. ## $P < 0.01$ indicates a significant difference from the Cy3G group.

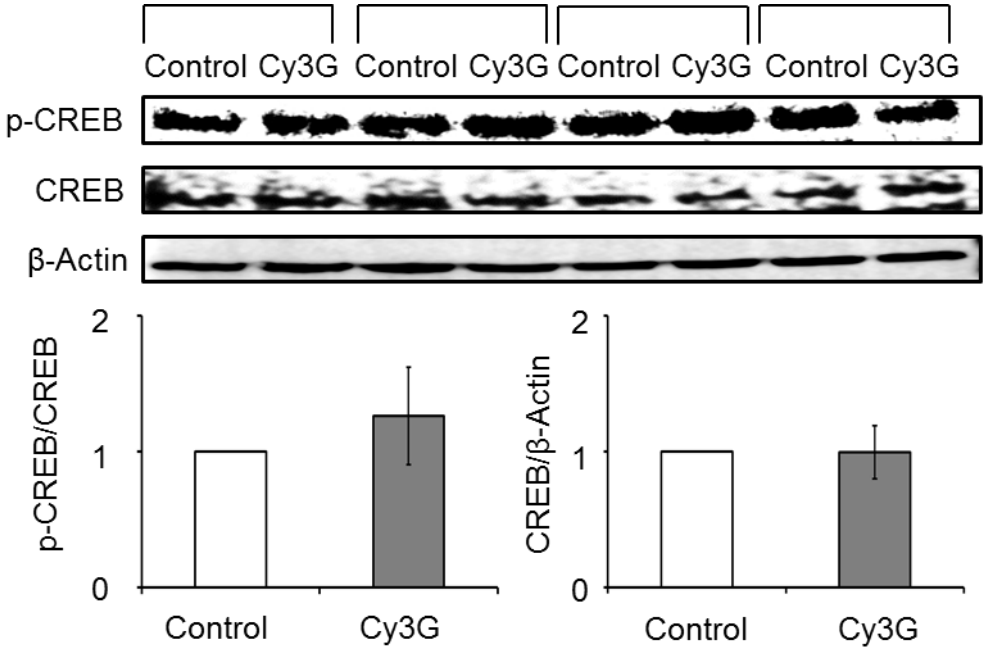


Figure 2-12. Effect of Cy3G on phosphorylation of CREB in C2C12 myotubes.
C2C12 myotubes were treated with or without Cy3G (10 μM) for 30 min and then phosphorylated CREB levels were evaluated and values were normalised to the β-actin expression level. All gels were run under the same experimental conditions and the representative blots were shown. Values are expressed as the mean ± standard deviation of triplicate experiments.

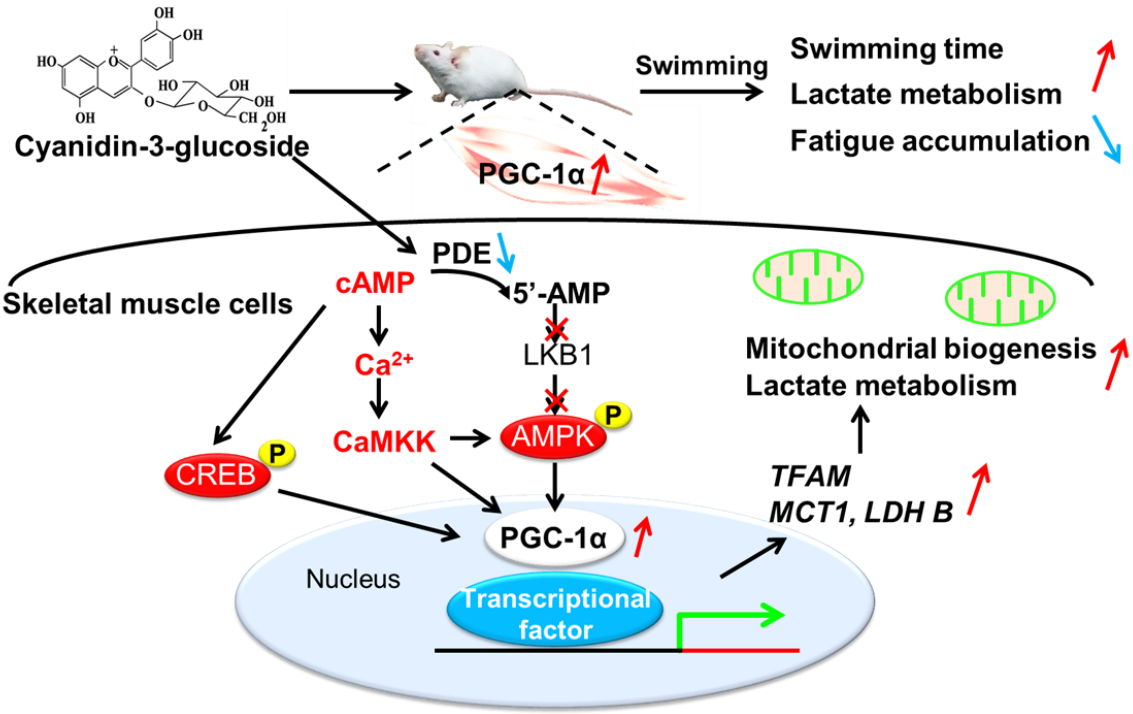


Figure 2-13. Suggested pathway for skeletal muscle PGC-1α upregulation by Cy3G enhances exercise performance.

Increase of lactate metabolism in response to Cy3G-induced PGC-1α upregulation enhanced exercise performance and reduced fatigue. This PGC-1α upregulation is modulated by CaMKK pathway via the elevation of intracellular cAMP levels.

Chapter 3

Elevated cAMP levels by cyanidin-3-glucoside prevents type 2 diabetes through inducing the formation of beige adipocytes phenotypes

Chapter 3. Elevated cAMP levels by cyanidin-3-glucoside prevents type 2 diabetes through inducing the formation of beige adipocytes phenotypes

The content of this chapter has been published in following papers with partial modifications:

Matsukawa T, Inaguma T, Han J, Villareal OM, Isoda H. Cyanidin-3-glucoside derived from black soybeans prevents type 2 diabetes through the induction of differentiation of preadipocytes into smaller and insulin-sensitive adipocytes. J Nutr Biochem 2015; 26: 860-867.

Matsukawa T, Villareal OM, Isoda H. The Type 2 Diabetes-preventive Effect of Cyanidin-3-glucoside on Adipocytes. J Dev Sus Agr 2016; 11: 31-35.

Matsukawa T, Villareal OM, Motojima H, Isoda H. Increasing cAMP levels of preadipocytes by cyanidin-3-glucoside treatment induces the formation of beige phenotypes in 3T3-L1 adipocytes. J Nutr Biochem 2017; 40: 77-85.

3.1 Introduction

The enhancement of metabolism is effective for improvement of metabolic diseases such as T2DM and obesity [32]. Chapter 2 discussed the effects of Cy3G on metabolism of skeletal muscle and exercise performance in models of exercised mice and cultured skeletal muscle cells. The major point discussed in Chapter 2 is that Cy3G enhances swimming time by increasing skeletal muscle PGC-1α expression through the elevation of cAMP levels (Fig. 2-13). PGC-1α plays an important role in the regulation of metabolism [24]. In individuals with metabolic diseases such as obesity, diabetes, and cardiomyopathy, PGC-1α expression in skeletal muscle is decreased as a result of increased inflammatory adipokines and FFAs [24, 76]. Therefore, results discussed in Chapter 2 predict that Cy3G may be effective for not only improved exercise performance, but also prevention of metabolic diseases since Cy3G can increase muscle PGC-1α expression. This chapter, the preventive effects of Cy3G against metabolic disease is discussed, focusing specifically on T2DM.

T2DM, one of the most common metabolic diseases, is a serious health problem, comprising 75~80% of all cases of diabetes with the number of patients increasing yearly around the world. The number of people afflicted with T2DM is estimated to reach 350 million by the year 2030 [78]. This is predicted to result in medical costs of treating diabetes to reach $336 billion by 2034 in the U.S. alone [79]. T2DM additionally raises various disease onset risks, such as cardiac infarction, atherosclerosis, stroke, and neurological disease [1, 80]. Insulin resistance (decreased sensitivity to insulin) is a pathognomonic symptom of T2DM wherein cells fail to respond to insulin, which leads to increased blood glucose levels following decreased glucose uptake ability of adipocytes and muscles [80]. It has also been reported that T2DM subjects have decreased metabolic activity since mitochondrial content and activity of skeletal muscle are reduced [74]. The improvement of metabolic environment (metabolic activity and insulin resistance) is therefore necessary for prevention of T2DM [80].

Adipose tissue plays a crucial role in the maintenance of metabolic processes such as the balance of energy, regulation of hormones and insulin sensitivity. In mammals, adipose tissue is mainly classified into two types, WAT and brown adipose tissues (BAT) (Table. 3-1) [81]. When energy intake exceeds energy consumption due to overeating, calorie-rich diet, sedentary lifestyle and aging, WAT stores excess energy as fat accumulation (increase in the number and/or size of adipocytes). As a result, secretion of inflammatory adipocytokines such as TNF-α, IL-6 and MCP-1 are increased, leading to decreased oxygen consumption, thermogenic activity, mitochondrial content and insulin sensitivity (Fig. 3-2) [27, 82]. In contrast to WAT, BAT plays an important role in metabolism (energy consumptions), especially thermogenesis [35]. Morphologically, brown adipocytes contain multilocular lipid droplets and numerous mitochondria [35]. Additionally, BAT express UCP-1, which is localized to the inner membrane of mitochondria and generates heat instead of ATP by diminishing the proton gradient [36]. BAT ablation or dysfunction in rodents induces obesity by decreasing energy expenditure [81]. Therefore, increasing energy expenditure by enhancing BAT function and number is an attractive means to combat lifestyle-related diseases [82]. However, BAT depots in human adults are small and present in defined places, such as the neck, interscapular region, and axillary region [82].

Brown-like adipocytes, called beige or brite (brown in white) adipocytes, have gained attention for regulation of whole-body energy expenditure [36]. Although their origin is the same as white adipocytes ($Myf5^-$ cells), beige adipocytes have characteristics of brown adipocytes, such as UCP-1 and PGC-1α expression, thermogenesis and higher numbers of mitochondria compared to white adipocytes [35, 36]. Furthermore, beige

adipocytes express several markers, such as CD137, T-box transcriptional factor (TBX1), transmembrane protein 26 (TMEM26), and Cbp/p300-interacting transactivator 1 (CITED1) [35, 36]. The increase in the number or activity of beige adipocytes in WAT is thought to be an effective way to maintain good health and/or prevent metabolic diseases since beige adipocytes can contribute to the regulation of whole-body energy expenditure like brown adipocytes [36]. Several external stimulations such as exercise, chronic cold exposure, norepinephrine, irisin, PPARγ agonists, and cAMP, have been shown to induce formation of beige adipocytes [75]. It has been reported that several dietary compounds such as fucoxanthin and capsaicin, also can induce formation of beige adipocytes [83].

Adipocyte differentiation (differentiation of preadipocytes into mature adipocytes) plays an important role in the development of brown and beige adipocyte phenotypes in mature adipocytes [84]. It has been reported that cAMP stimulation with early phase preadipocyte differentiation can induce beige adipocyte characteristics such as increased mitochondrial number and UCP-1 expression [85-87]. As shown in Fig. 2-11 B, intracellular cAMP levels were elevated in response to Cy3G treatment. Therefore, it is predicted that Cy3G may be able to induce the preadipocytes differentiation into beige adipocytes.

Black soybean (*Glycine max* (L.) Merr), called "Kuromame" in Japan, is widely recognized as a nutritionally rich food and has been used as folk medicine in East Asia for a long time. It is known that black soybean has higher anti-oxidative effects compared with yellow soybeans [20]. In Japan, specifically in Tanba and Mimasaka, which are famous black soybean producing districts, black soybeans extract is traditionally used to make black soybean tea, called as "Kuromame-cha" in Japan for folk medicine and is believed to be beneficial in the care of inflammatory and obesity-related diseases [88]. Black soybean seed coat is abundant in anthocyanins, especially Cy3G [89, 90]. It has been suggested that black soybean seed coat extract has a potential therapeutic effect against T2DM since administration of black soybean seed coat extract can ameliorate serum glucose concentration and insulin sensitivity in obese [88] and diabetic mouse models [91]. Kanamoto *et. al.* reported that black soybean seed coat extract (BSSCE) has potency in increasing UCP-1 expression in white adipose tissue [88]. Therefore, it is predicted that Cy3G contained in black soybeans may have the potential to induce formation of beige adipocytes. However, the effect of BSSCE and Cy3G on adipocyte differentiation is not fully understood. Furthermore, the effects of Cy3G on the induction of beige adipocytes and the relationship with T2DM also have not yet been reported. Here, effects of BSSCE and Cy3G on induction of beige adipocyte

phenotypes, T2DM in the db/db mouse, which is the standard T2DM mouse model, and 3T3-L1 cells as model of white adipocytes are discussed.

3.2 Materials & Methods

3.2.1. Preparation of black soybean seed coat extract

Black soybean (*Hitachi oguro*) was provided by Daigo town (Ibaraki, Japan). Its seed coats (1 g) were extracted with water (10 ml) at 105 ºC for 20 min. BSSCE was stored at -80 ºC until use.

3.2.2 Animal experiments

Five-week-old diabetic model BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J male mice (db/db mice) and lean mice were obtained from Charles River Laboratories (Kanagawa, Japan). The mice were housed at one mouse per cage and were maintained under a 12 h light/dark cycle and had free access to water and food. After one week of acclimatization, db/db mice were divided into two groups: Group 1 was orally administered with 30 mg/ kg of BSSCE while Group 2 was given only water (each group n=8 mice). Lean mice were also divided into two groups: Group 1 was orally administered with 30 mg/ kg of BSSCE every day while Group 2 was given only water (each group n=5 mice). The body weight of mice was measured daily and food intake was measured every week. After 30 days, mice were subjected to fasting for 20 h before sacrificed. The WAT samples (epididymis, perinephric, retroperitoneum and intestinal membrane) were weighed, then, fixed in 10% formalin at room temperature, frozen soaked for 2 h in 10% sucrose solution and embedded with O.T.C compound ESC21 and Cryomold 3 (SAKURA Finetek JAPAN Co.,Ltd) by dry ice/hexane immersion. The frozen block of samples was cut into 6 µm sections in -35 ˚C using Cryostats (Leica, CM1850, Japan) and photographs of the tissue sample crossections were taken using a microscope (Leica, DFC290 HD, Japan). All the procedures used in this study were approved by the International Animal Care and Use Committee and in compliance with the Guide for the Care and Use of Laboratory Animals of the University of Tsukuba.

3.2.3. Cell culture and differentiation of 3T3-L1 cells

Mouse 3T3-L1 cells (JCRB Cell Bank, Osaka, Japan) were cultured in Dulbecco's DMEM supplemented with 10% FBS and 1% penicillin (5000 µg/ml) – streptomycin (5000 IU/ml) at 37 ºC in a humidified atmosphere of 5% CO_2 using 75cm^2 flask. Cells at to 60~80% confluence were used for pass-culture. In each experiment, passaging number of 3~7 cells were used.

It has been reported that when preadipocytes differentiate into mature adipocytes, it gains the function for adipokines secretion and accumulation of lipid droplets [80]. Because of them, mature adipocytes, differentiated from 3T3-L1 preadipocytes were widely used

in vitro model of adipocytes. For differentiation into 3T3-L1 mature adipocytes, cells were seeded at 3×10^4 cells/cm^2 and cultured to reach confluence. Two days post-confluence (Day 0), cells were treated with differentiation medium (DMEM containing dexamethazone (DEX), 3-isobuthyl-1-methylxanthine (IBMX) and insulin) for 72 h 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). During the differentiation process (Day 0 to Day 7), 3T3-L1 cells were treated with or without BSSCE and Cy3G at the indicated concentrations (Fig. 3-2). DEX, IBMX and insulin solutions were purchased from Cayman Chemical Co., Ann Arbor, USA.

3.2.4. Measurement of lipid accumulation by Oil Red O staining

Lipid droplet in 3T3-L1 adipocytes were stained by Oil Red O (Merck Millipore,UK). Oil Red O (3 g/L) is dissolved by 100% Isopropanol (Wako, Japan). Differentiated 3T3-L1 adipocytes were fixed with 10% formaldehyde (Wako, Japan) for 15 min, and then washed by water. After that, cells were stained with 60% Oil Red O solution for 20 min and washed two times with water and one times with 60% Isopropanol. The after, morphology was observed under a light microscope. After taking the photography, stain solution was extracted using 100 μl/well of 100% Isopropanol for 15 min in Room temperature. After diluted, absorbance was measured at 490 nm using a Powerscan HT plate reader. The value was calculated as a percentage of control.

3.2.5. Measurement of cell viability by MTT assay

The cell viability was determined by 3- (4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MTT were purchased from Dojindo, Japan and dissolved to 5 mg/ml in PBS and stored at 4℃. MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes (mitochondrial reductase). The content of MTT indicates the cellular mitochondrial activity and cell number. Therefore, MTT assay was widely used to evaluate the cell viability.

3T3-L1 cells were seeded in 96-well plate (1.0×10^4 cells/well) and were differentiated into mature adipocytes as described above. 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 100 μl/well of 10% SDS (Wako, Japan) followed by overnight incubation (around 16 h). After that, the absorbance was detected at 570 nm using a Powerscan HT plate reader. The values were normalized to the value of the growth medium and calculated as a percentage (%) of control.

3.2.6. Quantification of mRNA levels by Real-Time PCR

Total RNA isolation and TaqMan real-time PCR amplification reactions were performed as previously described in Chapter 2. For the quantification of gene expression, the following TaqMan probes purchased from Applied Biosystems were used: *β-actin* (Mm00607939_s1), *PPARγ* (Mm01184322_m1), *C/EBPα* (Mm00514283_s1), *GLUT4* (Mm00436615_m1), *FABP4* (Mm00445878_m1), *PGC-1α* (Mm01208835_m1), *SIRT1* (Mm00490758_m1), *UCP-3* (Mm00494077_m1), *TFAM* (Mm00447485_m1), *CytC* (Mm01621048_s1), *PDK4* (Mm01166879_m1), *SOD2* (Mm01313000_m1), *UCP-1* (Mm01244861_m1), *UCP-2* (Mm00627599_m1), *TBX1* (Mm00448949_m1), *CITED1* (Mm01235642_g1), and *C/EBPβ* (Mm00843434_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).

3.2.7. Measurement of adiponectin and TNF-α secretion in 3T3-L1 adipocytes

The quantification of the adiponectin and TNF-α concentrations in 3T3-L1 adipocytes culture medium was performed using Adiponectin ELISA kit (Otsuka Pharma., Co., Ltd., Tokyo, Japan) and TNF-α ELISA kit (R&D Systems, Minneapolis, MN), respectively, according to manufacturer's instructions. Absorbance was measured at 450 nm using a Powerscan HT plate reader. Adiponectin and TNF-α concentrations were calculated using the standard curve and values were expressed as a percentage (%) of control.

3.2.8. Cell culture and differentiation of C2C12 myoblasts

The culture and differentiation procedure of mouse C2C12 myoblasts is Described in Chapter 2.

Briefly, after differentiation, C2C12 myotubes were treated with conditioned medium, which is the Cy3G-treated 3T3-L1 culture medium, or control (untreated with Cy3G) for 24 h.

3.2.9. Quantification of protein levels by Western blotting

Protein extraction and western blotting were performed as previously described in Chapter 2. Briefly, protein samples (15 μg) were separated using 10 % SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked using Odyssey blocking buffer for 1 hour, and incubated with the following antibodies: Akt (1:10000 dilution), phospho-Akt (Ser473) (1:10000 dilution), phospho-Insulin receptorβ (Tyr1146) (1:10000 dilution), Insulin receptorβ (1:10000 dilution), and β-Actin (1:5000 dilution), at 4°C

overnight, then incubated with secondary antibodies: IRDye 800CW donkey anti-rabbit IgG (1:10000 dilution) or IRDye 680LT goat anti-mouse (1: 20000 dilution) (LI-COR, Inc., NE, USA) at room temperature for 30 min. Akt, phospho-Akt (Ser473), phospho-Insulin receptorβ (Tyr1146), and Insulin receptorβ were purchased from Cell Signaling Technology (Hertfordshire, UK). The signal was detected using the OdysseyFc Imaging System. All protein quantifications were normalized to the β-Actin expression level.

3.2.10. Measurement of glucose uptake level

Glucose uptake was measured in differentiated 3T3-L1 cells using 2-Deoxyglucose Uptake Measurement Kit (Cosmo bio, Tokyo, Japan) according to the manufacturer's instructions. Absorbance was measured at 420 nm using a Powerscan HT plate reader. Glucose uptake levels were expressed as a percentage (%) of control.

3.2.11. Measurement of the mitochondrial content by rhodamine 123

Mitochondria content in 3T3-L1 adipocytes was measured using a Rhodamine 123 as previously described in Chapter 2.

Briefly, 3T3-L1 cells were seeded in 96-well plate at 1.0×10^4 cells/well and were differentiated into mature adipocytes as described above. After the differentiation, 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. After the incubation, fluorescent images were obtained using a BZ-X710 All-in-One fluorescence microscope (Keyence, Osaka, Japan). Hoechst 33342 solution (Dojindo, Kumamoto, Japan) was used to stain nucleus. 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. Rosiglitazone (Wako, Tokyo, Japan) was used as positive control of increasing of mitochondrial content.

3.2.12. Measurement of intracellular ATP level

The intracellular ATP level in 3T3-L1 adipocytes were measured using "Cellno" ATP ASSAY reagent as previously described in Chapter 2.

Briefly, 3T3-L1 cells were seeded at 1.0×10^4 cells/well and were differentiated into mature adipocytes as described above. After differentiation, ATP assay reagents were added to each well (100 μl/well) and incubated for 10 min at 25 ºC. After that, the

luminescence was measured using a Powerscan HT plate reader. The values were calculated as a percentage (%) of control.

3.2.13. Measurement of intracellular ROS level

Intracellular reactive oxygen species (ROS) level was measured using a fluorescent dye, 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA was purchased from Enzo Life Sciences (Lausen, Switzerland). Differentiated 3T3-L1 adipocytes (Day 7) were cultured in serum-free DMEM containing 10 μM DCFH-DA at 37 ºC 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.

3.2.14. Measurement of intracellular cAMP level

The intracellular cAMP level in 3T3-L1 cells were measured using the cAMP-Glo^{TM} MAX assay 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. Two days post-confluence (Day 0), cells were cultured in serum-free DMEM containing 20 mM $MgCl_2$ with or without PDE inhibitors (500 μM IBMX and 100 μM Ro20-1724) and with or without Cy3G (100 μM) for 30 min. Then, the luminescence was measured using a Powerscan HT plate reader. Intracellular cAMP levels were calculated based on a standard curve and values were expressed as a percentage (%) of control. In this assay, PDE inhibitors were used to prevent cAMP hydrolysis during the assay.

3.2.15. Statistical analysis

All the results were expressed as the mean ± SD, and the statistical evaluation was performed using Student's t-test when two value sets were compared. When multiple comparisons were carried out, one way ANOVA followed by Tukey's multiple comparison test was performed using SPSS (IBM Statistics for Windows, version 22.0. IBM Corp, Armonk, NY). $P<0.05$ was considered to be statistically significant.

3.3 Results

3.3.1. The increase of WAT weight and size of db/db mice were prevented by BSSCE

To explore the preventive effect of BSSCE on T2DM progression, db/db mice were given BSSCE for 30 days. As shown in Fig. 3-3 A, the body weight of untreated db/db mice gradually increased over time. However, BSSCE blocked the increase in body weight compared with the control (control: 11.1 g vs BSSCE group: 5.6 g). Furthermore, total WAT (epididymis, perinephric, retroperitoneum, and intestinal membrane) weight in db/db mice was significantly decreased by BSSCE supplementation (Fig. 3-3 B). Reduced size of WAT is one of the characteristics of beige adipocytes [35, 36]. As shown in Fig. 3-4 A and B, the size of WAT in db/db mice was decreased by BSSCE. However, the body weight and total WAT of lean mice was not statistically difference between control and BSSCE-supplemented mice (Table 3-2).

3.3.2. Cy3G induced smaller and multilocular lipid droplets in adipocytes by promoting adipocytes differentiation without cytotoxicity

Adipocyte differentiation (differentiation of preadipocytes into mature adipocytes) plays an important role in the development of brown and beige adipocyte phenotypes in mature adipocytes [84]. 3T3-L1 cells can differentiate into mature adipocytes and are widely used to study differentiation and metabolism in adipocytes [92]. To evaluate the effect of Cy3G on adipocyte differentiation, 3T3-L1 preadipocytes were treated with BSSCE and Cy3G during the differentiation process (Day 0 to Day 7) (Fig. 3-2). BSSCE-treated preadipocytes differentiated into adipocytes containing smaller and multilocular lipid droplets compared with control cells (Fig. 3-5 A, B and C). Black soybean seed coat is known to be rich in anthocyanins, including Cy3G [89, 90]. Cy3G-treated 3T3-L1 adipocytes also showed smaller and multilocular lipid droplets (Fig. 3-5 D and E). Furthermore, as shown in Fig. 3-6 A, Cy3G (20 to 100 μM) does not show cytotoxicity in 3T3-L1 adipocytes. These results indicate that Cy3G is the main active compound in BSSCE and that it can induce preadipocytes to differentiate into smaller and adipocytes containing multilocular lipid droplets without cytotoxicity. Additionally, Cy3G (100 μM) increased the expression of *PPARγ* and *CCAAT/enhancer-binding protein α* (*C/EBPα*), important transcriptional factors for the regulation of adipocyte differentiation, by 3.5- and 3.4-fold, respectively (Fig.3-7 B). Accompanying adipocyte differentiation, the expression of adipocytes markers, such as glucose transporter type 4 (GLUT4), fatty acid binding protein 4 (FABP4), and adiponectin, are increased by PPARγ and C/EBPα [93]. As shown in Fig. 3-6 B, Cy3G increased *GLUT4* and *FABP4* expression by 2.3- and 2.4-fold, respectively. Therefore, these results suggest that Cy3G induces smaller

and multilocular lipid droplet in adipocytes by promoting differentiation.

3.3.3. Cy3G increased adiponectin secretion and deceased TNF-α secretion from 3T3-L1 adipocytes

WAT plays an important role in maintaining insulin sensitivity and metabolic activity by releasing of adipokines [33, 34]. TNF-α reduces insulin sensitivity and metabolic activity while adiponectin activates their [33, 34]. It has been suggested that smaller and multilocular lipid droplets adipocyte have a higher ratio of adiponectin/TNF-α secretion compared to bigger and unicolor lipid droplets adipocyte [33, 34]. As show in Fig. 3-7, adiponectin production was increased, while TNF-α production decreased with Cy3G treatment. In the human body, adipokines, such as adiponectin and inflammatory cytokines, influence the metabolic activity and insulin sensitivity of skeletal muscles (Fig. 3-8). Increasing PGC-1α expression and activity in skeletal muscle by adiponectin signalling promotes glucose uptake, mitochondrial biogenesis and oxidative capacity [94]. To test this idea, C2C12 myotubes (*in vitro* skeletal muscle model cells) treated with conditioned medium from Cy3G-treated 3T3-L1 adipocyte cultures for 24 h. As shown in Fig. 3-9, the expression of *PGC-1α*, *SIRT1*, and *UCP-3* were increased by Cy3G treatment 195%, 140%, and 237%, respectively. Elevation of these genes in C2C12 myotubes correlated with increased adiponectin and decreased TNF-α secretion.

3.3.4. Cy3G increased the expression of insulin receptor and glucose uptake levels in 3T3-L1 adipocytes

Insulin resistance is a main symptom of T2DM causing cells to fail to respond to normal actions of insulin and leading to decreased glucose uptake by adipocytes [78]. To evaluate the effect of Cy3G on glucose uptake in 3T3-L1 adipocytes, the proteins in the insulin signaling pathway and glucose uptake levels were evaluated. As shown in Fig. 3-10, phosphorylation of the insulin receptor (IR) and Akt were not increased in Cy3G-treated 3T3-L1 adipocytes. However, the expression of IR and Akt were increased by 1.7-fold and 1.8-fold, respectively. Additionally, Cy3G increased glucose uptake ability of 3T3-L1 adipocytes (Fig. 3-11). These results suggest that Cy3G enhances glucose uptake of 3T3-L1 adipocytes through increased IR expression.

3.3.5. Cy3G increased the expression of *UCP-1* in 3T3-L1 adipocytes

Smaller and multilocular lipid droplets are morphological characteristics of beige adipocytes [35]. Beige adipocytes have characteristics similar to brown adipocytes, such as UCP-1 expression [35, 36]. Cy3G increased the gene expression of *UCP-1* (Fig.

3-12 A). UCP-1 plays important roles in thermogenesis and decreasing the production of ROS by diminishing the proton gradient rather than synthesizing ATP [95]. As shown in Fig. 3-11 B and C, intracellular ROS and ATP production were decreased in Cy3G-treated 3T3-L1 adipocytes.

3.3.6. Cy3G increased the mitochondrial content and mitochondrial genes expression in 3T3-L1 adipocytes

Increased mitochondrial number and the expression of mitochondrial genes are known to be important markers of brown and beige adipocytes [35]. Therefore, the effect of Cy3G on mitochondrial content and the expression of mitochondrial genes in 3T3-L1 adipocytes were evaluated. As shown in Fig. 3-13 A and B, 50 and 100 μM 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 increase in the mitochondrial content by Cy3G was almost same as by rosiglitazone, which has been reported to induce beige adipocyte phenotypes [84, 96]. TFAM is known to be a key regulator of mitochondrial biogenesis by regulating the replication and transcription of the mitochondrial genome [97]. The expression of *TFAM* and mitochondrial genes (*CytC*, *PDK4*, *UCP-1*, *UCP-2*, and *SOD2*) were elevated by Cy3G treatment (Fig. 3-12 A). These results indicate that Cy3G increased mitochondrial content and mitochondrial genes expression.

3.3.7. Cy3G induced the expression of *TBX1* and *CITED1* during the adipocytes differentiation process

Beige adipocytes show not only the same characteristics as brown adipocytes, but also express several specific markers, such as CD137, TBX1, and CITED1 [35, 36]. In fact, CITED1, one of the beige adipocytes selective markers, is known to be expressed in UCP-1 positive beige adipocytes [98]. As shown in Fig. 3-14 A, Cy3G increased the expression of beige adipocyte selective markers, *TBX1* and *CITED1*. Therefore, these results suggest that Cy3G induced beige adipocyte phenotypes in 3T3-L1 adipocytes. It has been proposed that the regulation of differentiation of preadipocytes is an important step in the development of brown and beige adipocyte phenotypes in mature adipocytes [84]. As show in Fig. 3-13 B and C, *TBX1* and *CITED1* elevation by Cy3G were detected as early as three days of treatment. Furthermore, *CITED1* expression was most highly increased at Day 3 (Fig. 3-14 C). PPARγ and C/EBPα are important transcriptional factors to regulate both white and brown adipocyte differentiation [37, 99]. During the adipocyte differentiation process, C/EBPα expression is enhanced in response to treatment with a cocktail of differentiation hormones (DEX, IBMX, and insulin) for 2-

3 days [100]. As shown in Fig. 3-15, *PPARγ* and *C/EBPα* expression were enhanced by Cy3G both in the presence and in the absence of the differentiation cocktail after three days of induction (Day 3). Therefore, these results suggest that Cy3G promotes the expression of beige adipocyte markers by regulating the preadipocytes differentiation.

3.3.8. Cy3G increased *C/EBPβ* expression and the intracellular cAMP levels

C/EBPβ, widely recognized as a crucial transcriptional factor for preadipocyte differentiation, induces the transcription of PPARγ and C/EBPα [99, 100]. C/EBPβ expression is rapidly and transiently increased by treatment with differentiation hormonal cocktail [99, 100]. In the presence of the differentiation hormonal cocktail, the expression of *C/EBPβ* was significantly enhanced by 1 and 3 hr Cy3G treatment to 1.9- and 3.2-fold, respectively (Fig. 3-16 A). Interestingly, Cy3G also increased *C/EBPβ* expression by 1.8-fold in the absence of the differentiation hormonal cocktail (Fig. 3-16 B). The expression of C/EBPβ is induced by intracellular cAMP levels through activation of CREB [101]. As shown in Fig. 3-15 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. 3-16 C).

3.4 Discussion

The key finding of this chapter is that Cy3G induces the formation of beige adipocyte phenotypes by increasing the intracellular cAMP levels (Fig. 3-17). Around the world, T2DM is one of the most serious health problems facing society, comprising 75–80% of all cases of diabetes [78]. T2DM raises numerous additional disease onset risks, such as cardiac infarction, atherosclerosis, stroke, and neurological disease [1, 80]. The improvement of metabolic environments, including both energy and hormonal balance, and insulin sensitivity, are important for prevention of T2DM [78, 80]. The adipose tissue plays a crucial role in the maintenance of metabolic environments (balance of energy, hormones balance and insulin sensitivity). When energy intake exceeds energy consumption, WAT stores excess energy as fat accumulation, which leads to increased secretion of inflammatory adipocytokines, eventually leading to the development of decreased oxygen consumption, thermogenic activity, mitochondrial content, and insulin sensitivity [27, 82]. Beige adipocytes appearing in WAT play an important role in the regulation of energy expenditure since they have similar characteristics to brown adipocyte [35, 36]. Therefore, an increase in the number or activity level of beige adipocytes in WAT could be an effective way to prevent metabolic diseases [36]. This chapter finding suggests that Cy3G may be an effective therapeutic agent for the prevention and treatment of T2DM and other metabolic diseases.

Body weight gain is a major risk factor in the development of T2DM because it induces insulin resistance and decreases metabolism activity [78, 80]. Hypertrophic adipocytes, store excessive fat (increase in the number and/or size of adipocytes), which lead to increased secretion of inflammatory adipocytokines and FFA, all of which lead to the development of insulin resistance [5, 33]. Furthermore, leptin resistance is observed in obese and diabetic individuals and is associated with reduced suppression of food intake (increased appetite) and decreased energy consumption [5, 102]. Leptin deficient (db/db mice) are widely used as a model of metabolic diseases such as obesity, diabetes, and dyslipidemia. It has been reported that db/db mice show an abnormal deposition of fat at 3 to 4 weeks of age and their increase in body weight reached a plateau at about 10 weeks [103]. In this chapter, data from the study of db/db mice orally administered BSSCE from 6 to 10 weeks resulting in reduced total body and WAT weight gain compared with control db/db mice are presented (Fig. 3-3 A and B). Therefore, these results suggested that BSSCE may prevent the progression of T2DM. BSSCE also reduced food intake of db/db mice (Table 3-2). However, food intake of BSSCE-fed lean mice was not reduced (Table 3-2). This suggests that the taste was not the

underlying reason for the observed decrease in food intake of db/db mice. It has been reported that black soybean anthocyanin-fed Sprague-Dawley rats showed decreased expression of neuropeptide Y (NPY), a hypothalamic modulator of appetite, accompanied with decreased body weight gain and food intake [104]. It is predicted that reduced NPY expression in the hypothalamus may also contribute to reduction of food intake in BSSCE-administrated db/db mice.

The increase in the number or activity level of beige adipocytes in WAT may be an effective way to maintain good health or prevent metabolic diseases since beige adipocytes can contribute to the regulation of whole-body energy expenditure like brown adipocytes [36]. Smaller and multilocular lipid droplets are the morphologically specifications of beige adipocytes [35]. Differentiation of preadipocytes into mature adipocytes plays an important role in the inducing of beige adipocyte phenotypes in mature adipocytes [84]. Correlated with WAT weight, the size of adipocytes in the WAT was also decreased in BSSCE-fed db/db mice (Fig. 3-3 and -4). Furthermore, BSSCE- and Cy3G-treated 3T3-L1 adipocytes also showed smaller and multilocular lipid droplets (Fig. 3-5). In the current study, BSSCE and Cy3G treatment were administered during the differentiation process (Day 0 to Day 7) (Fig. 3-2). It is known that black soybean seed coat is rich in anthocyanins, specifically Cy3G [89, 90]. Pharmacokinetic studies revealed that absorbed Cy3G reaches not only the plasma, but also the adipose tissues in glycoside form through the circulation systems [21, 22]. Therefore, these results suggest that Cy3G has beneficial effects on adipocytes by preventing hypertrophy and favoring differentiation of preadipocytes into smaller and multilocular lipid droplets containing adipocytes. Through the experiments presented in this chapter, it was found that Cy3G induced similar characteristics as brown adipocyte, such as an increase of multilocular lipid droplets, mitochondrial content and *UCP-1* expression, as well as expression of beige adipocyte markers in 3T3-L1 adipocytes (Fig. 3-12 and -13). It is already known that BSSCE has potency in increasing UCP-1 expression in WAT [90]. Therefore, it is predicted that Cy3G contained in black soybeans induces the formation of beige adipocytes in WAT.

Increased mitochondrial ROS production causes the synthesis of faulty proteins, oxidized lipids and mtDNA mutations, which leads to decreased metabolic activity and cellular insulin sensitivity [105]. Correlated with decreases in the intracellular ROS production in Cy3G-treated adipocytes, the expression levels of genes associated with ROS elimination in mitochondria, such as superoxide dismutase (SOD) and UCP, were increased (Fig. 3-12 A). For instance, SOD regulates intracellular ROS levels as ROS scavenging enzyme [105]. UCP-1, a marker of brown and beige adipocytes, plays an

important role in thermogenesis and decreases the ROS produced by diminishing the proton gradient instead of synthesizing ATP [95]. Furthermore, UCP-2, which is a mitochondrial anion carrier protein present in the mitochondrial inner membrane of WAT, also controls ROS production to protect against oxidative stress [106]. This suggests that decreased intracellular ROS production in Cy3G-treated adipocytes is associated with the upregulation of *SOD-2*, *UCP-1*, and *UCP-2*, which are related to reduction of ROS production. Increased intracellular ROS production induces insulin resistance (inhibition of insulin signaling), a key pathological feature of T2DM, through the activation of c-Jun NH_2 terminal kinase (JNK) and IκB kinase [107]. Therefore, it is predicted that regulation of these genes may partially be involved in the preventative effects of BSSCE on T2DM progression.

The AMPK pathway is activated by depleted ATP levels (increasing of AMP/ATP ratio) and promotes mitochondrial biogenesis, glucose uptake, and β-oxidation [108]. Overexpression of UCP-1 in adipocytes causes an increase in the ratio of AMP/ATP, eventually activating AMPK, since UCP-1 dissipates the proton gradient of the mitochondrial membrane to produce heat (thermogenesis) instead of ATP production [36, 109]. Activated AMPK increases the expression of TFAM, which is a key regulator of mitochondrial biogenesis [108]. *TFAM* upregulation was observed corresponding with increased mitochondrial content (Fig. 3-12). Therefore, it is most likely that the activation of AMPK in response to mitochondrial uncoupling explains the increase of mitochondria content in Cy3G-treated adipocytes. Accompanied with adipocyte differentiation, mitochondrial biogenesis and oxidative capacity are induced because the adipocyte differentiation process requires a large amount of ATP during this process [110, 111]. Cy3G was found to increase mitochondrial content and the expression of mitochondrial oxidative genes, such as *CytC* and *PDK4* (Fig. 3-11 A and -12). These results suggest that Cy3G promotes adipocyte differentiation as well as increases mitochondrial biogenesis and oxidative capacity.

Differentiation of preadipocytes into mature adipocytes is an important process for the regulation of metabolic environments such as adipokine secretion, insulin sensitivity, and metabolic activity [93]. PPARγ and C/EBPα are transcription factors that regulate numerous adipocyte-related genes and are involved in both white and brown adipocytes differentiation [37, 93]. Recent studies revealed that chronic PPARγ agonist treatment or prolonged triiodothyronine, IBMX, and rosiglitazone treatment when preadipocytes are differentiating into mature adipocytes can induce brown and beige adipocyte-associated gene expression in mature adipocytes. These studies suggested that the regulation of differentiation of preadipocytes is a crucial step for the development

of brown and beige adipocytes phenotypes in mature adipocytes [84, 112]. In this chapter, data showing that Cy3G treatment during the differentiation process also induces beige adipocyte marker expression is presented (Fig. 3-14). The current results also support the idea that the regulation of differentiation is an important step for enhancing the induction of beige adipocyte phenotypes in mature adipocytes. PPARγ agonists induce the formation of beige and brown adipocytes phenotypes by stabilizing the PR domain-containing protein-16 (PRDM16) and increasing early B-cell factor-2 (EBF2) expression [84, 113]. Polyphenols, such as phloretin [114] and flavanone [115], exhibit PPARγ agonist activity and promote adipocyte differentiation by increasing PPARγ transcriptional activity. Although, upregulation of *PPARγ* expression by Cy3G was observed (Fig. 3-6 B), PPARγ agonist activity of Cy3G was not detected (data not shown). The upregulation of *PPARγ* without PPARγ agonist activity also promotes adipocyte differentiation [116, 117]. Therefore, it is predicted that the mechanisms of inducing the formation of beige adipocytes phenotypes by Cy3G are different from those of PPARγ agonist.

PPARγ and C/EBPα, are transcriptional factors that regulate adipokine secretion and glucose metabolism by regulating the expressions of genes associated with adipocytes differentiation [118, 119]. For example, adiponectin, which has the function of activating insulin sensitivity and metabolic activity, is regulated by transcriptional activity of PPARγ [120, 121]. Furthermore, C/EBPα is involved in acquiring insulin sensitivity through the regulation of IR and GLUT4 expression [118, 122]. Consistent with *PPARγ* and *C/EBPα* upregulation, both adiponectin production and the expression of IR in Cy3G-treated adipocytes were increased (Fig. 3-7 B and -10). Therefore, these results suggest that Cy3G increases glucose uptake ability by *PPARγ* and *C/EBPα* upregulation.

C/EBPβ is widely recognized as an important transcriptional factor for preadipocyte differentiation since it induces the transcription of PPARγ and C/EBPα [101]. In committed preadipocytes, C/EBPβ expression is very low but it is rapidly and transiently enhanced by differentiation hormonal stimulation, specifically via cAMP stimulation [101]. As shown in Fig. 3-16 B and C, intracellular cAMP elevation was induced before the *C/EBPβ* upregulation (cAMP: 30 min treatment, C/EBPβ: 60 min treatment). Therefore, the elevation of *C/EBPβ* is induced by intracellular cAMP elevation in response to Cy3G treatment and these phenomena are indicators of promotion of preadipocyte differentiation (Fig. 3-16). Moreover, C/EBPβ also plays an important role in inducing PGC-1α and UCP-1 expression by interacting with PRDM16 during early differentiation and in combination with cAMP stimulation to induce white preadipocytes

to develop characteristics of beige adipocytes [85-87]. Together, these data suggest that the elevation of intracellular cAMP levels by Cy3G induces the transcription of beige adipocytes-associated genes and eventually induces the differentiation of white preadipocyte into beige adipocytes (Fig. 3-18). In this study, the role of Cy3G in increasing the expression of two beige adipocyte makers, CITED1 and TBX1 is revealed. As shown in Fig. 3-13, the increasing rate of *CITED1* is higher than that of *TBX1* at all of measurement points (Day 3, 5, and 7). Additionally, *CITED1* expression was most highly increased at Day 3. It has been reported that CITED1 expression is increased by intracellular cAMP through protein kinase A (PKA) [123]. On the other hand, increased TBX1 expression is associated with transforming growth factor-beta (TGF-β) signaling pathways [124]. As shown in Fig. 3-15 C, Cy3G induces intracellular cAMP elevation. Therefore, it is thought that *CITED1* expression is strongly increased by Cy3G, compared with *TBX1* expression.

In the body, intracellular cAMP levels are regulated by PDEs, which are key enzymes that hydrolyze cAMP to 5′AMP [74]. As shown in Fig. 3-16 C, in the presence of PDE inhibitors, Cy3G increased intracellular cAMP levels, and this effect was abrogated by the absence of PDE inhibitors. Based on in vitro cell-free assay results, Cy3G was shown to have PDE inhibitory effects [75]. Therefore, these results suggest that Cy3G increases the intracellular cAMP levels in preadipocytes through PDE inhibition.

In conclusion, the oral administration of BSSCE prevented phenotypes associated with the progression of T2DM, such as the gain of WAT weight and size. Additionally, *in vitro* studies revealed that Cy3G induces the 3T3-L1 adipocytes to attain beige adipocyte characteristics (multilocular lipid droplets, increased mitochondrial content, *UCP-1* expression and beige adipocyte markers). Furthermore, Cy3G promoted preadipocytes differentiation by increasing of *C/EBPβ* through the elevation of the intracellular cAMP. These results indicate that elevated cAMP levels by Cy3G induce beige adipocytes phenotypes in 3T3-L1 adipocytes, all of which ultimately prevent the progression of T2DM (Fig. 3-17). The results of this chapter suggest that Cy3G may be used as an effective therapeutic agent for prevention of T2DM through improvement of metabolic environments.

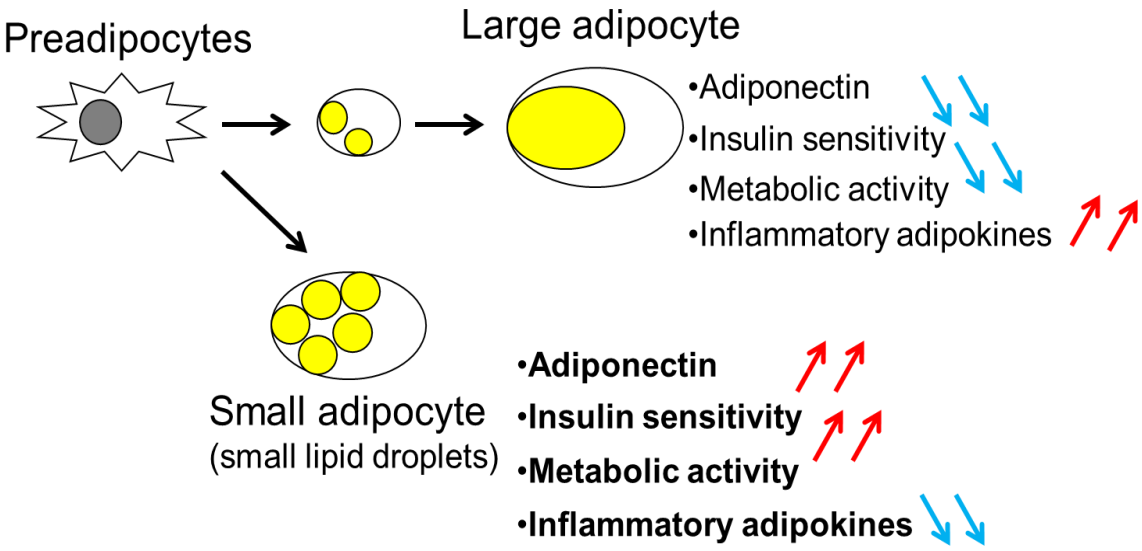
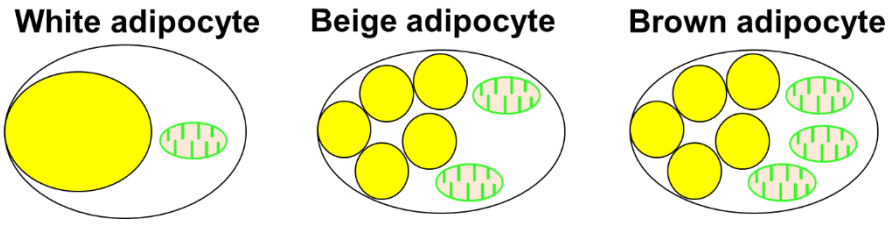


Figure 3-1. Schematic diagram of adipocytes differentiation.

Preadipocytes differentiate into mature adipocytes. Large adipocytes, are contained large lipid droplets, show decreased adiponectin secretion and increased free fatty acids and inflammatory adipokines secretions which leads to lowered insulin resistance and metabolic activity. On the other hands, small adipocytes, are contained multilocular lipid droplets, have higher insulin sensitivity, metabolic activity, and adiponectin levels as well decrease inflammatory regulators' secretion.



	White	Beige (Brown-like)	Brown
Location	Mesenteric Omental Subcutaneous	Inguinal Subcutaneous	Interscapular Perirenal Axillary
Morphology	Unilocular Large lipid droplets	Multiple small lipid droplets	Multiple small lipid droplets
Mitochondria	Low (+)	++	+++
Function	Storage of energy as triglycerides	Thermogenesis	Thermogenesis
Origin	$Myf5^-$ cells	$Myf5^-$ cells	$Myf5^+$ cells
Enriched markers	LPL, G3PDH, Resistin	CD137, TMEM26, TBX1, CITED1, UCP-1, PGC-1α	PDK4, EVA1, EBF3, UCP-1, PGC-1α
Activators	High Fat Diet	Cold, FGF21, Irisin Thiazolidinediones	Cold, FGF21, Irisin Thiazolidinediones, BMP7, Orexin

Table. 3-1: Differences among the three types of adipocytes.

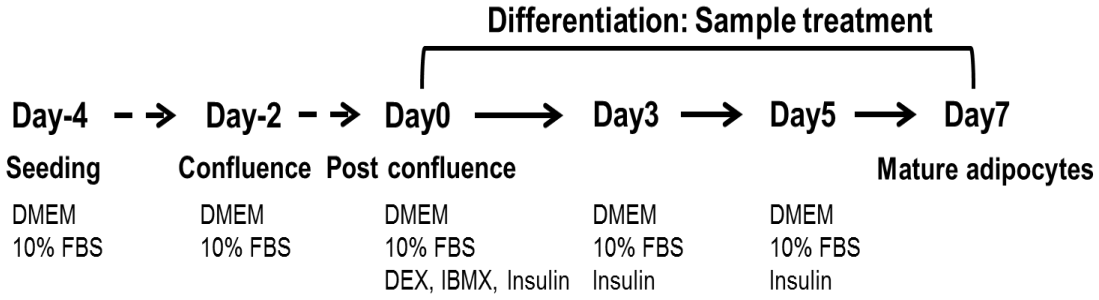


Figure 3-2: Experimental procedure of differentiation of 3T3-L1 adipocytes.
For differentiation into 3T3-L1 mature adipocytes, cells were seeded and cultured to reach confluence. Two days post-confluence (Day 0), cells were treated with differentiation medium (DMEM containing DEX, IBMX and insulin) for 72 h 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). During the differentiation process (Day 0 to Day 7), 3T3-L1 cells were treated with sample.

Table 3-2. Effect of the body weight, food intake and Total WAT weight in lean and db/db mice.

	lean mice		db/db mice	
	Control	BSSCE	Control	BSSCE
Initial body weight (g)	22.5 ± 0.87	24.2 ± 0.76	31.0 ± 1.0	30.0 ± 1.0
Final body weight (g)	23.0 ± 1.22	23.2 ± 1.75	42.2 ± 2.56	35.7 ± 1.25*
Food intake (g/week)	23.2 ± 3.61	24.6 ± 0.48	43.9 ± 3.86	33.7 ± 2.37**
Total WAT weight (g)	0.75 ± 0.12	0.59 ± 0.05	4.62 ± 0.38	2.44 ± 0.23**

Values are expressed as the mean±SD. * $P<0.05$, ** $P<0.01$ indicates the mean value that is significantly different from that of the control group in db/db mice.

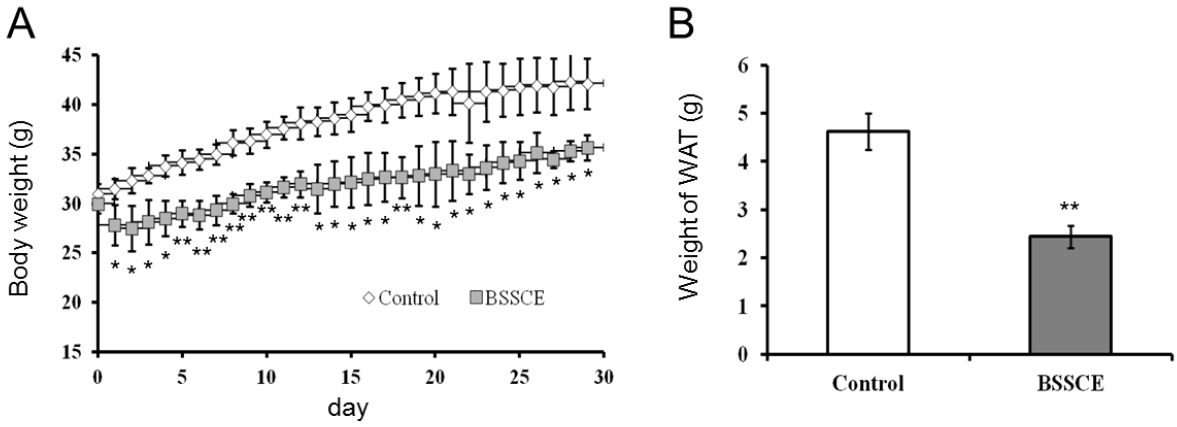
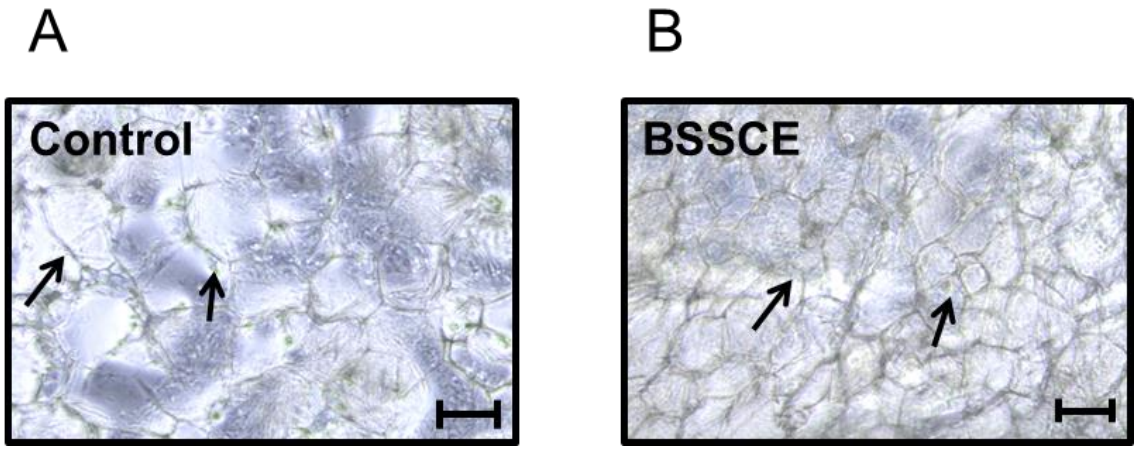


Figure 3-3. Effect of BSSCE on the gain of the body weight and white adipose tissue (WAT) of db/db mouse.

BSSCE (30 mg/kg) or water (Control) was orally administered everyday for 30 days in db/db mice. The body weight (g) of db/db mice was measured everyday (A) and after 30 days, the WAT weight (g) was determined (B). Values are expressed as the mean±SD. * *P<0.05*, ** *P<0.01* indicates the mean value that is significantly different from that of the control group.



Figures 3-4. Effect of BSSCE on the size of white adipose tissue in db/db mice.
BSSCE (30 mg/kg) or water (Control) was orally administered everyday for 30 days in db/db mice. (A and B) Histological sections of white adipose tissues of Control (A) and BSSCE group (B). Scale bar indicates 200 μm.

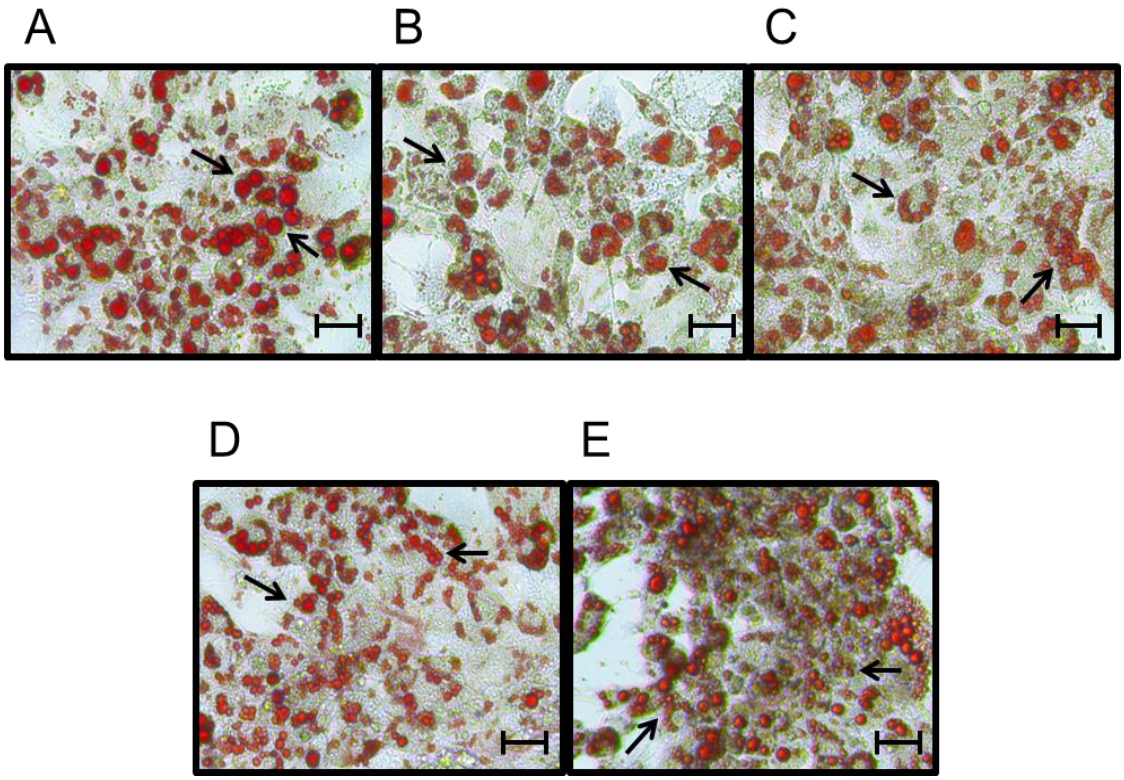


Figure 3-5. Effect of BSSCE and Cy3G on the differentiation of 3T3-L1 preadipocytes into adipocytes.

3T3-L1 preadipocytes were treated with or without BSSCE or Cy3G during the differentiation process for 7 days. After differentiation, cells were stained with Oil Red O. Control (A), Cy3G-containing BSSCE at 20 μM (B) or 100 μM (C), Cy3G at 20 μM (D) or 100 μM (E). Arrow indicates lipid droplets. Scale bar indicates 100 μm.

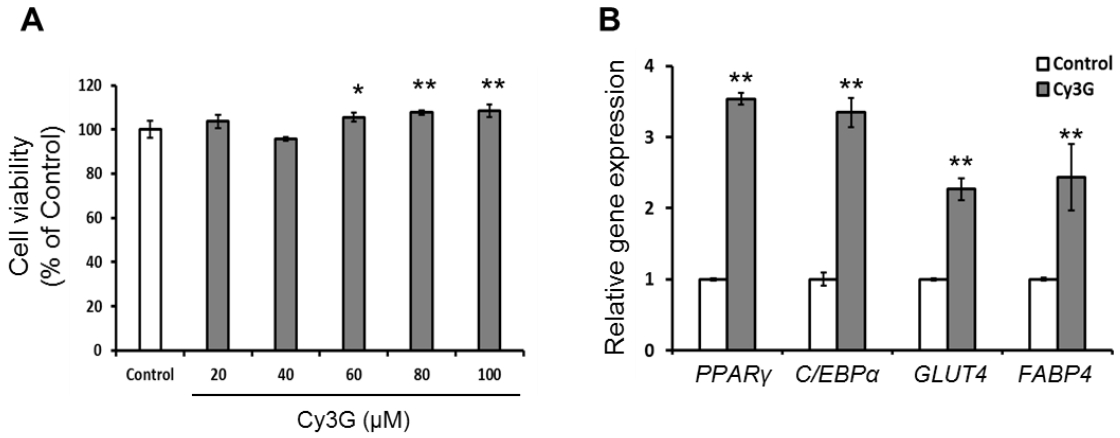


Figure 3-6. Effect of Cy3G on the expression of mature adipocytes markers.
During the differentiation process (Day 0 to Day 7), 3T3-L1 preadipocytes were treated with or without 20 to 100 μM. After differentiation, cell viability (A), and gene expression levels of mature adipocytes markers (*PPARγ*, *CEBPα*, *GLUT4*, and *FABP4*) (B), were evaluated. (A) Cell viability was determined by MTT assay. Values are expressed as a percentage (%) of the control. (B) Gene expression levels were normalized to *β-actin* expression levels. (A and B) Results are expressed as the mean ± SD of triplicate experiments. * $P<0.05$, ** $P<0.01$ indicates the mean value is significantly different from that of the control.

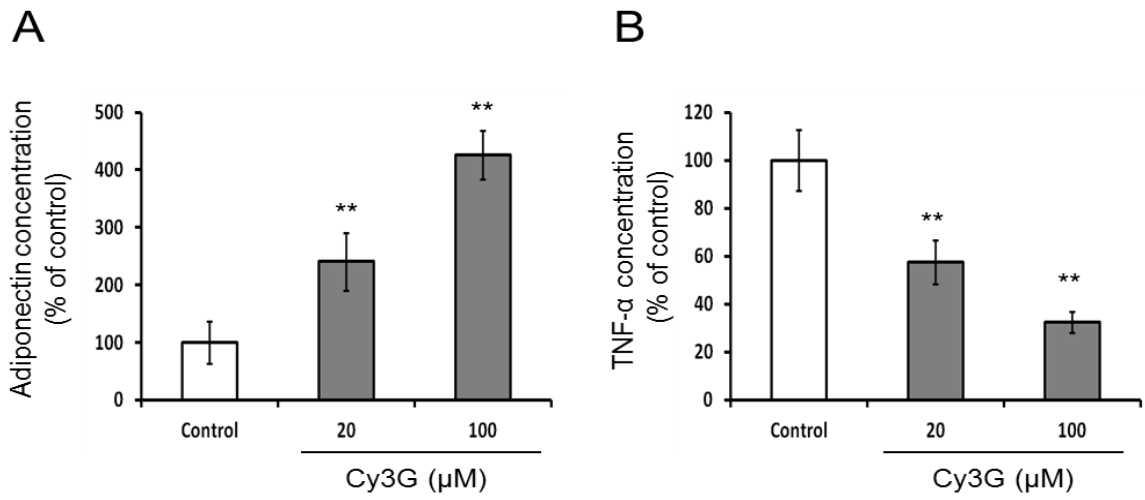


Figure 3-7. Effect of Cy3G on adiponectin and TNF-α secretion in 3T3-L1 adipocytes.

3T3-L1 preadipocytes were treated with or without Cy3G during the differentiation process for 7 days. After differentiation, adiponectin (A) and TNF-α (B) secretion levels in 3T3-L1 adipocyte were evaluated by ELISA assay. Values are expressed as the mean ± SD of triplicate experiments. ** *P<0.01* indicates the mean value that is significantly different from that of the control group.

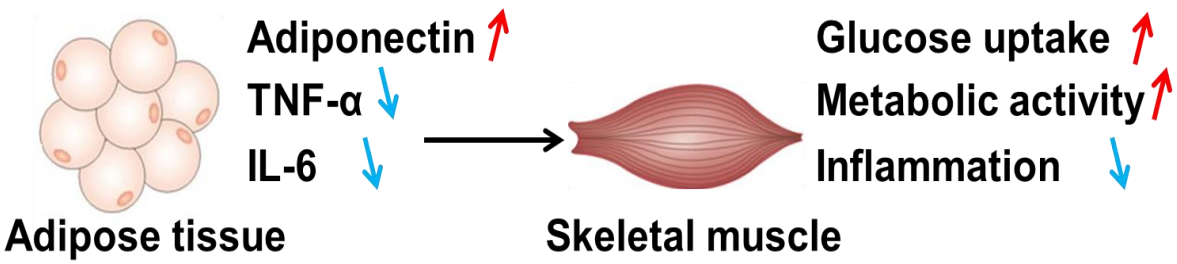


Figure 3-8. Schematic diagram of influence of adipokines on the metabolic activity and insulin sensitivity of skeletal muscle.

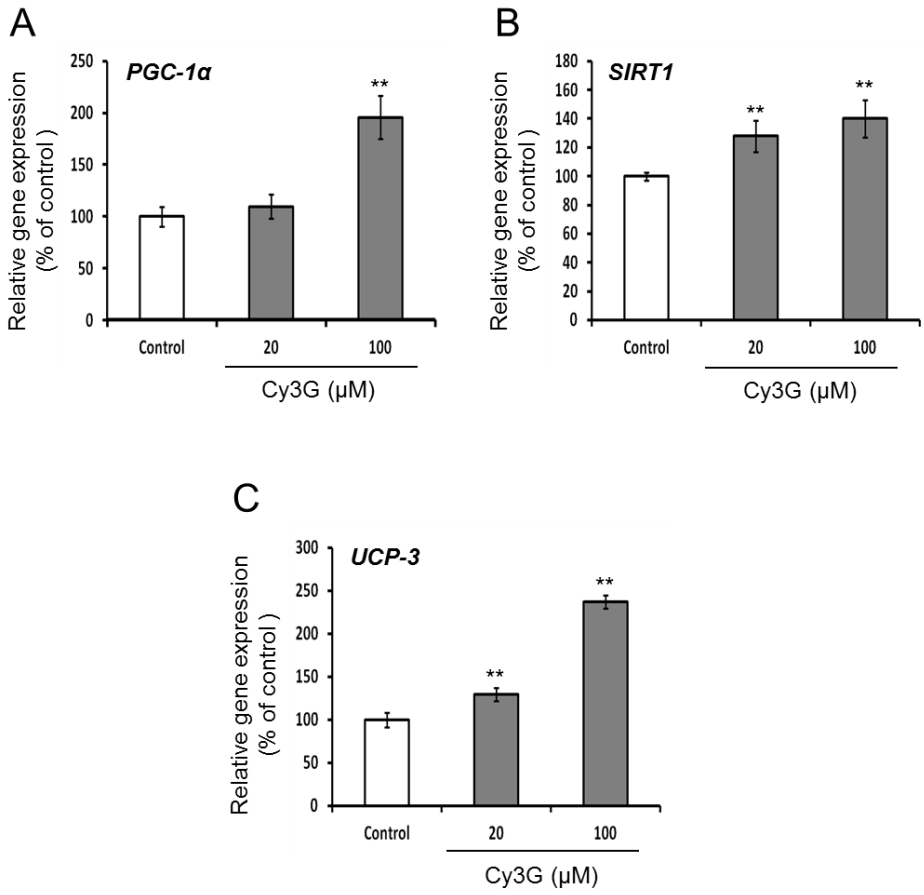


Figure 3-9. Effect of Cy3G-treated 3T3-L1 culture medium on the gene expression of *PGC-1α*, *SIRT1*, and *UCP-3* in C2C12 myotubes.

3T3-L1 preadipocytes were treated with or without Cy3G during the differentiation process for 7 days. After differentiation, C2C12 myotubes cultured with condition medium obtained from Cy3G-treated 3T3-L1 culture medium or control (untreated with Cy3G) for 24 h. After that, the expression levels of *PGC-1α* (A), *SIRT1* (B) and *UCP-3* (C) were evaluated. Values were normalized to the *β-actin* expression level are expressed as the mean ± SD of triplicate experiments. ** $P<0.01$ indicates the mean value that is significantly different from that of the control group.

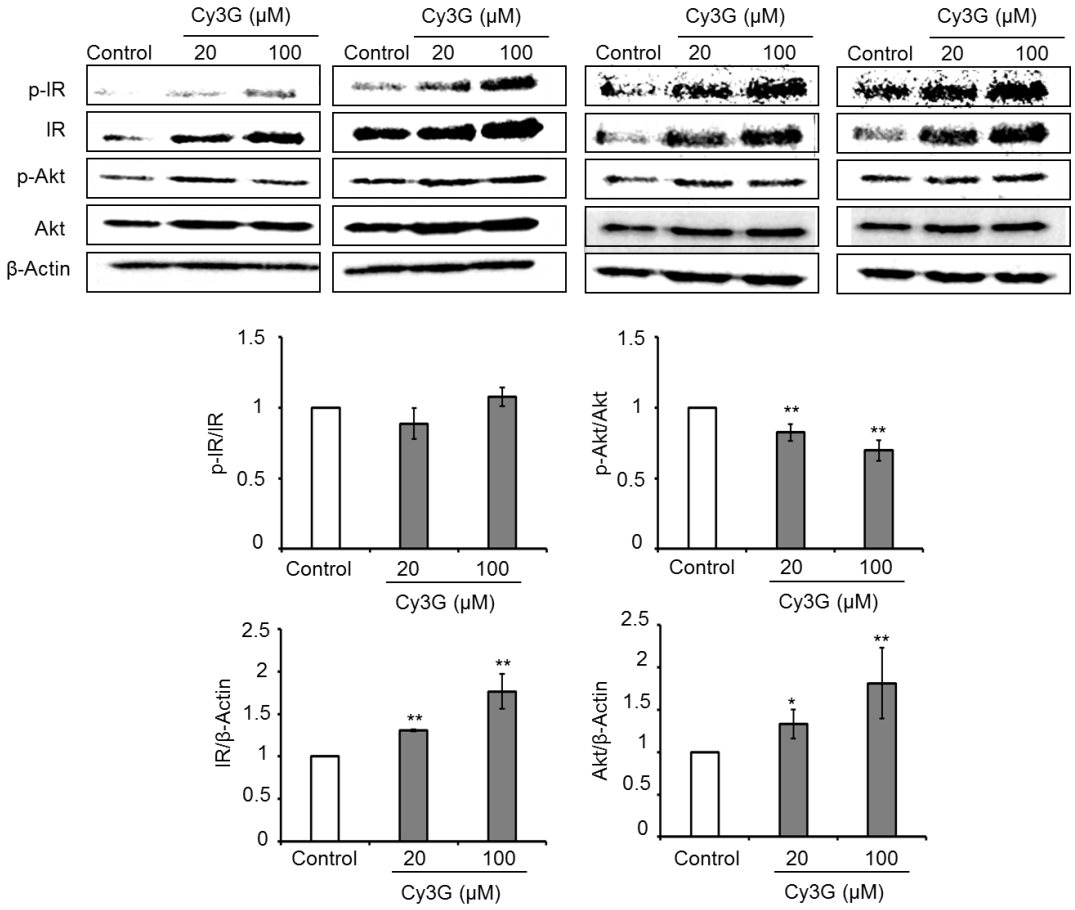


Figure 3-10. Effect of Cy3G on the expression of insulin receptor and Akt in 3T3-L1 adipocytes.

3T3-L1 preadipocytes were treated with or without Cy3G during the differentiation process for 7 days. Differentiated adipocytes were cultured in serum-free DMEM (3 h) and adipocytes were treated with 100 nM insulin (10 min). After that, the expression of phosphorylated insulin receptor (IR) and Akt and non-phosphorylated IR and Akt were evaluated. Values are expressed as the mean ± SD of triplicate experiments. * *P<0.05*, ** *P<0.01* indicates the mean value that is significantly different from that of the control group.

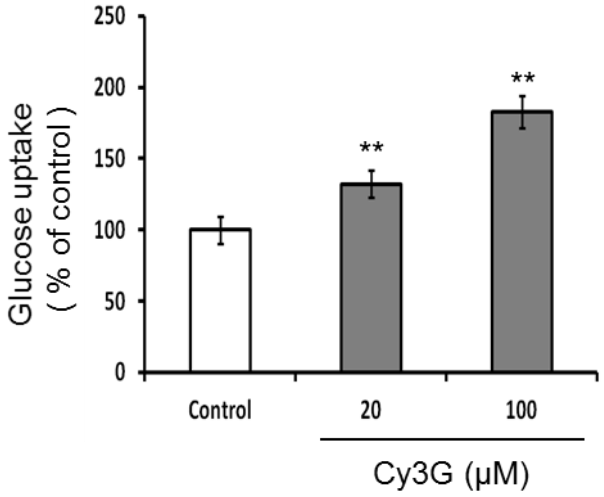


Figure 3-11. Effect of Cy3G on glucose uptake levels in 3T3-L1 adipocytes.
3T3-L1 preadipocytes were treated with or without Cy3G during the differentiation process for 7 days. After differentiation, glucose uptake level were evaluated. Values are expressed as the mean ± SD of triplicate experiments. ** *P<0.01* indicates the mean value that is significantly different from that of the control group.

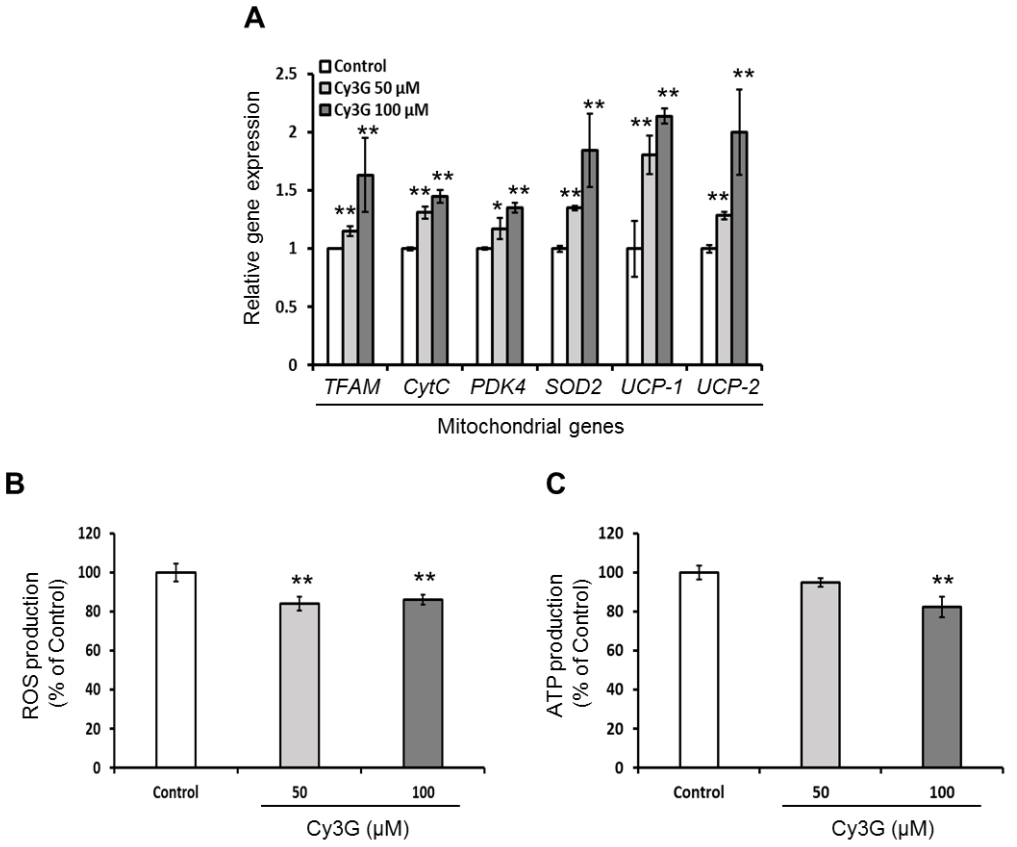


Figure 3-12. Effect of Cy3G on the expression of mitochondrial genes in 3T3-L1 adipocytes.

3T3-L1 preadipocytes were treated with or without 50 and 100 μM Cy3G during the differentiation process for 7 days. After differentiation, mitochondrial gene (*TFAM*, *CytC*, *PDK4*, *SOD2*, *UCP-1*, and *UCP-2*) expression (A), and intracellular ROS (B) and ATP production (C) in 3T3-L1 adipocytes were evaluated. (A) Expression levels of mRNA were normalized to the *β-actin* expression level. (B and C) Values were expressed as a percentage (%) of the control. Results are expressed as the mean ± SD of triplicate experiments. * $P<0.05$, ** $P<0.01$ indicates the mean value is significantly different from that of the control.

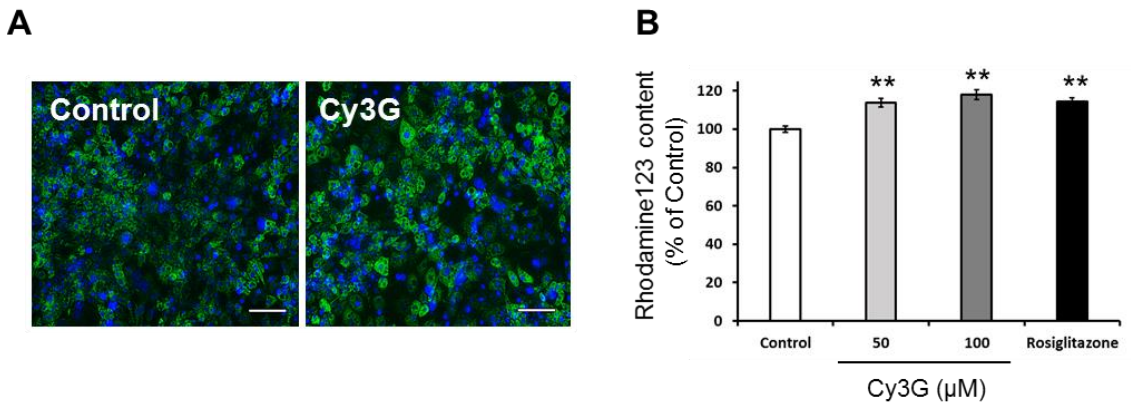


Figure 3-13. Effect of Cy3G on mitochondrial content in 3T3-L1 adipocytes.
3T3-L1 preadipocytes were treated with or without 50 and 100 μM Cy3G during the differentiation process for 7 days. After differentiation, mitochondrial content in 3T3-L1 adipocytes were evaluated. Mitochondria were stained by a mitochondrial specific dye, Rhodamine 123. Photographs are on the Control (Left) and Cy3G (100 μM) (Right). Rhodamine 123 (mitochondria) is green and Hoechst 33342 (nuclei) is blue. Scale bar indicates 100 μm. To quantify the rhodamine 123 content, cells were lysed using 1% Triton X-100 and fluorescence intensity (485 nm/528 nm) was measured. Values were expressed as a percentage (%) of the control. Results are expressed as the mean ± SD of triplicate experiments. * P<0.05, ** P<0.01 indicates the mean value is significantly different from that of the control.

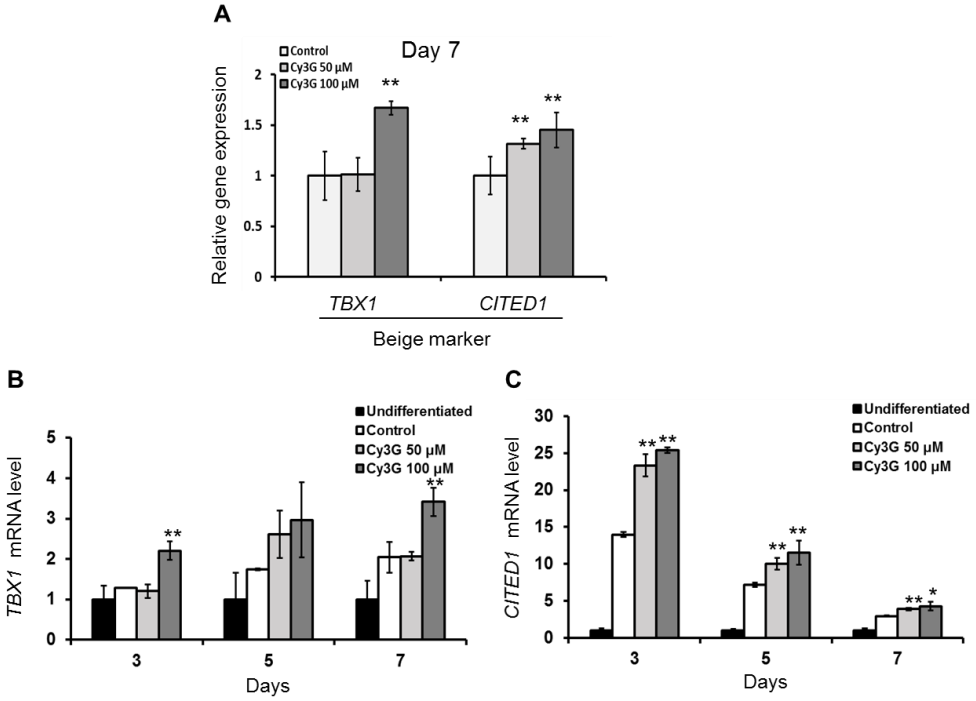


Figure 3-14. Time dependent effect of Cy3G on *TBX1* and *CITED1* expression in 3T3-L1 cells.

3T3-L1 preadipocytes were treated with or without 50 and 100 μM Cy3G during the differentiation process for 3, 5, and 7 days. After differentiation (Day 7), beige adipocyte markers (*TBX1* and *CITED1*) in 3T3-L1 adipocytes were evaluated (A). The change of *TBX1* and *CITED1* expression in 3T3-L1 cells during the differentiation process on Day 3, 5, 7 days are shown (B and C). Values were normalized to the *β-actin* expression levels and expressed relative to the undifferentiated 3T3-L1 cells. Results are expressed as the mean ± SD of triplicate experiments. * $P<0.05$, ** $P<0.01$ indicates the mean value is significantly different from that of the control.

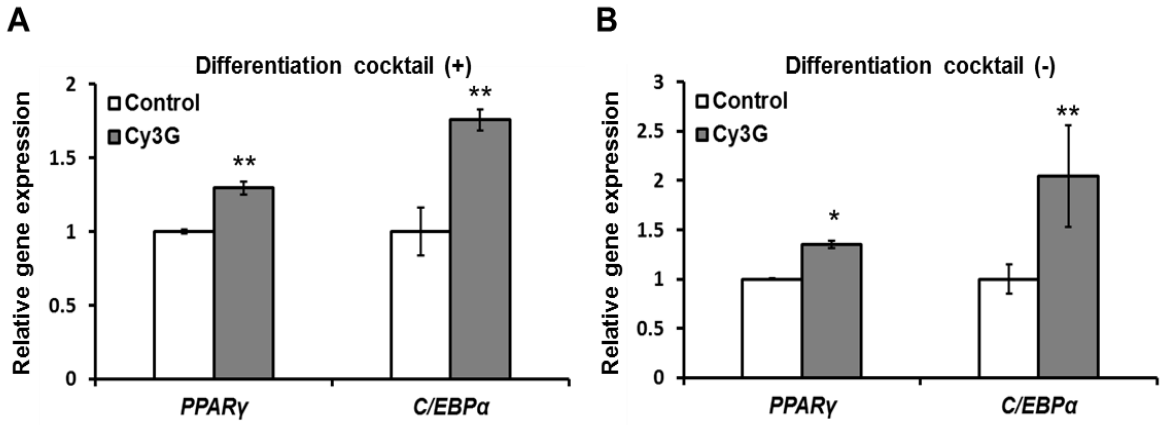


Figure 3-15. Effect of Cy3G on *PPARγ* and *C/EBPα* expression at early stage of differentiation.

3T3-L1 preadipocytes (Day 0) were treated with or without Cy3G (100 μM) 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 *PPARγ* and *C/EBPα* were evaluated. Values are normalized to the *β-actin* expression levels and expressed as the mean ± SD of triplicate experiments. ** $P<0.01$ indicate the mean value that is significantly different from that of each control groups.

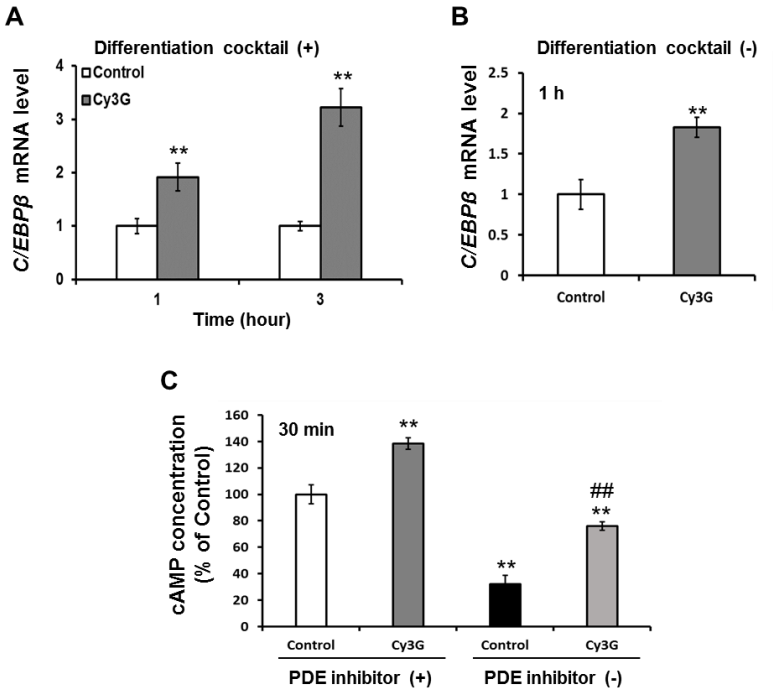


Figure 3-16. Effect of Cy3G on *C/EBPβ* expression and intracellular cAMP levels in 3T3-L1 cells at early stage.

(A) 3T3-L1 preadipocytes (Day 0) were treated with or without Cy3G (100 μM) in the presence of a differentiation hormonal cocktail for 1-3 h. (B) 3T3-L1 preadipocytes were treated with or without 100 μM Cy3G in the absence of the differentiation hormonal cocktail for 1 h. After treatment, *C/EBPβ* mRNA levels were evaluated. (C) 3T3-L1 preadipocytes were treated with or without Cy3G (100 μM) and with or without PDE inhibitor for 30 min. After that, the intracellular cAMP level was measured and values were expressed as a percentage (%) of the control. Results are expressed as the mean ± SD of triplicate experiments. ** $P<0.01$ indicates significant difference compared to the Control. ## $P<0.01$ indicates significant difference compared to the Cy3G (PDE inhibitor +). Multiple comparisons were carried out using ANOVA followed by Tukey's multiple comparison test.

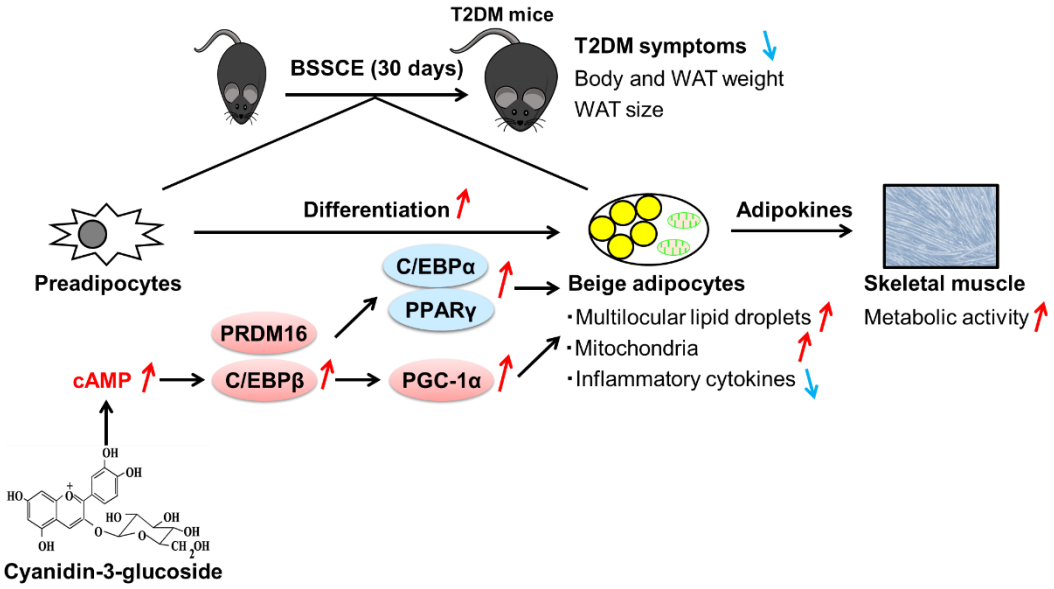


Figure 3-17. Suggested pathway for cyanidin-3-glucoside prevented the progression of T2DM by inducing of beige adipocytes phenotypes through promoting adipocyte differentiation.

Cy3G prevented the progression of T2DM by inducing of beige adipocytes phenotypes through promoting adipocyte differentiation. Promotion of adipocyte differentiation is induced by the elevation of the intracellular cAMP level.

Chapter 4

General Discussion

Chapter 4: General Discussion

Modern lifestyle and environmental factors often adversely affect human metabolism, causing metabolic as well as hormonal and nutritional imbalances. These in turn lead to a predisposition to various lifestyle-related diseases, including obesity and T2DM, by affecting our metabolic environment, such as unbalance of metabolism, hormone, and nutrition [1, 2]. In fact, more than two thirds of deaths in Japanese populations are reported to be due to lifestyle-related diseases, or "seikatsusyukanbyo," as they are known in Japan [2]. These lifestyle-related diseases are not only a problem in their own right, but also increase the risk of developing additional lifestyle-related diseases; therefore, their prevention and/or amelioration is critical for prolonged human health. Recently, nutraceuticals, such as dietary fiber, probiotics, vitamins, and polyphenols, have gained attention as alternatives to conventional pharmaceuticals, since they promote the proper maintenance of a healthy metabolic environment via the regulation of both energy and hormone balance [7]. This thesis focused on Cy3G, a polyphenol compound present in numerous colorful fruits and vegetables such as black soybean, blueberry, and grapes [125], and evaluated both its effects on the metabolic environment, and the underlying regulatory mechanisms through which these effects are exerted.

Chapter 2 of this thesis investigated the effects of Cy3G on both exercise performance, and the metabolic activity of the skeletal muscle, by using exercised mice and cultured skeletal muscle cells as model systems. In a previous study, Cy3G was shown to increase the expression of *PGC-1α*, encoding a transcriptional coactivator essential for the regulation of metabolism, in skeletal muscle cells [23]. Skeletal muscle is considered the organ that has the greatest impact on the regulation of energy balance within the body, since it accounts for 40% of basal metabolic function [38, 39]. Additionally, elevated muscle metabolism and function contribute to exercise performance [32]. As demonstrated in Chapter 2, increasing muscle lactate metabolism in response to Cy3G-induced PGC-1α upregulation enhanced swimming time and reduced fatigue. Furthermore, *PGC-1α* upregulation was shown to occur via the CaMKK pathway through the elevation of intracellular cAMP levels. Decreased muscle *PGC-1α* expression has been previously shown to be associated with several lifestyle-related diseases [24]. Therefore, it is likely that Cy3G may enhance exercise performance and prevent and/or ameliorate lifestyle-related disease, by increasing *PGC-1α* expression in the muscle and thereby regulating energy balance. This hypothesis is

supported by the results presented in chapter 3, which showed Cy3G to exert a preventive effect against T2DM. Regular exercise is effective in ameliorating various lifestyle-related diseases by positively regulating the metabolic environment in the body [32]. Myokines, (i.e. cytokines and peptides released from skeletal muscle), regulate the metabolism of organs such as the adipose tissue, liver, pancreas, and brain [126]. Thus, it is likely that Cy3G may prevent lifestyle-related diseases not only via its direct effects, but also by enhancing exercise quality. To establish the validity of this hypothesis, future investigation is required to establish whether modulating both Cy3G activity and exercise can synergistically prevent lifestyle-related diseases.

Chapter 3 presented the effects of Cy3G on the induction of beige adipocytes, and the corresponding effect of this induction on the prevention of metabolic diseases using a T2DM mouse strain, and the 3T3-L1 cell line, as model systems. T2DM is a serious health condition, and comprises 75–80% of all diabetes cases [80]. The adipose tissue plays a crucial role in the maintenance of healthy metabolic environments, including both energy and hormonal balance, and insulin sensitivity. Recently, the appearance of beige adipocytes in WAT has gained attention, since these adipocytes are established to mediate energy expenditure. In fact, it is predicted that increasing either the number, or activity level of beige adipocytes in WAT, may be an effective method for preventing various metabolic diseases [36]. Several external stimuli such as exercise, chronic cold exposure, norepinephrine, irisin, PPARγ agonists, and cAMP, have been shown to induce beige adipocyte phenotypes [79]. In this study, Cy3G has been shown to increase intracellular cAMP levels in skeletal muscle cells, and thus, it is hypothesized that Cy3G may be able to induce the differentiation of preadipocytes into beige adipocytes. This is supported by the results presented in chapter 3, which showed that the elevation of intracellular cAMP levels in response to Cy3G-treatment induced beige adipocyte characteristics associated with the prevention of T2DM (i.e. multilocular lipid droplets, increased mitochondrial content, UCP-1 expression, and beige adipocyte markers), in 3T3-L1 adipocytes (Fig. 3-18). In the body, beige adipocytes have been shown to be derived from inguinal and subcutaneous adipocytes [35, 36]. Whilst the present study demonstrated the ability of Cy3G to induce the beige adipocytes phenotypes from 3T3-L1 adipocytes, the 3T3-L1 cell line is mouse-derived, and thus, further research is needed to clarify the effects Cy3G treatment on human inguinal and subcutaneous adipocytes. Nevertheless, the results presented in chapter 3 suggest that Cy3G may be an effective therapeutic target for the prevention of T2DM and other metabolic diseases, via the activation of metabolic environments and the regulation of energy and hormonal balances. The differentiation of preadipocytes into mature adipocytes is crucial to ensure the correct

regulation of metabolic functions including adipokine secretion, insulin sensitivity, and metabolic activity [93]. Thiazolidinediones (TZDs), such as troglitazone and pioglitazone, are PPARγ agonists used to treat T2DM, and can increase insulin sensitivity by promoting adipocyte differentiation [127, 128]. While TZDs exert therapeutic effects against diabetes, they also induce adverse effects on the body, such as weight gain and edema. In chapter 3, Cy3G was shown to induce beige adipocyte characteristics by promoting adipocyte differentiation via the elevation of intracellular cAMP, without PPARγ agonist activity. Furthermore, body weight gain caused by BSSCE was not observed in either lean or db/db mice treated with Cy3G. Thus, the induction of beige and insulin-sensitive adipocytes by Cy3G may be an effective therapy for the prevention and/or treatment of T2DM, which induces only a low-level of side effects compared to treatment with TZDs.

This thesis evaluated the effects of Cy3G, and the underlying regulatory mechanisms by which these effects were exerted. Several pharmacokinetic studies have previously reported that absorbed Cy3G reaches not only the plasma, but also several other tissues, as a glycoside [21, 22, 129]. In contrast, some glycoside compounds are converted to aglycones during absorption by the small intestine, suggesting that it may be important to examine the effects of cyanidin in the context of Cy3G. Several previous studies have reported cyanidin to exert beneficial effects on the body, including anti-inflammatory, anti-obesity, and anti-oxidative effects [130, 131]. Interestingly, Tsuda *et. al.* showed Cy3G to be capable of enhancing the expression of *PPARγ*, (this being essential to enable adipocyte differentiation), whist cyanidin was not [116, 117, 130]. As described in chapter 3, the promotion of preadipocyte differentiation is crucial for Cy3G-induced beige adipocytes phenotypes. Thus, further study needed but the results presented in this thesis suggest that Cy3G may be suitable for inducing beige adipocytes phenotypes compared to cyanidin. Furthermore, Zou *et. al.* observed Caco-2 cells to absorb Cy3G through GLUT2, a glucose transporter expressed in the small intestine [132]. Adipocytes and skeletal muscle cells exhibit high expression of a related glucose transporter isotype, GLUT4. Further study is needed to establish whether Cy3G permeates cell membranes via GLUT4.

The primary finding of this thesis is that Cy3G can increase intracellular cAMP. PDEs are key enzymes that hydrolyze cAMP to 5′AMP, thereby regulating intracellular cAMP levels [74]. In the cAMP assay conducted during the current study, PDE inhibitors were used to prevent cAMP hydrolysis. Cy3G was shown to increase intracellular cAMP levels in the presence of these PDE inhibitors, and this effect was abrogated in the absence of the inhibitors. A previous study by Dallas *et. al.* showed

Cy3G to inhibit PDEs in *in vitro* cell-free assays [75]. Together, these results suggest that Cy3G increases intracellular cAMP levels via PDE inhibition. Flavonoids have been reported to also be potent inhibitors of PDEs, and flavonoid-mediated PDE inhibition has been demonstrated to be dependent upon the ability of a flavonoid to sterically fit into the cyclic nucleotide binding pocket, for example, His-529, His-563, Asp-564, and Asp-674 [133, 134]. Peluso reported that the position of hydroxylation (-OH) in flavonoids is important for PDE inhibition, such as that shown for the addition of hydroxyl groups at C-3, C-5, C-7, and C-4' [134, 135]. Cy3G has several hydroxyl groups incorporated into its structure at C-3, C-5, and C-4', that are predicted to mediate its PDE-inhibitory activity. Catechins, which lack a double bond between positions 2 and 3 of the typical C-ring structure displayed by cyanidin, does not exhibit PDE-inhibitory activity [135]. In contrast, kaempferol and quercetin, both of which have four hydroxyl groups at C-3, C-5, C-7, and C-4', exhibit high PDE-inhibitory activity, although the effects of kaempferol and quercetin on adipocytes are different from those exerted by Cy3G [135]. Kaempferol and quercetin not only suppress lipid accumulation by inhibiting adipocytes differentiation but also inhibits glucose uptake ability in adipocytes [136]. Of these effects, suppressed lipid accumulation is effective against both obesity and T2DM. In contrast, the inhibition of glucose uptake ability is not favorable in T2DM, since this disease is characterized by high blood-glucose levels [78, 80]. Together, these results suggest that Cy3G may be more suitable for the prevention of T2DM and/or obesity than either kaempferol or quercetin.

The second common key finding of this thesis is that Cy3G can increase PGC-1α expression. PGC-1α is a transcriptional coactivator established to play a crucial role in the regulation of metabolic environments, for example, such as maintaining metabolic and hormonal balance [24]. The expression of PGC-1α is downregulated in several lifestyle-related diseases such as obesity, diabetes, and cardiomyopathy, and therefore, it is predicted that a Cy3G-induced increase in PGC-1α expression may contribute to the management of a variety of lifestyle-related diseases. This study evaluated the effects of Cy3G on adipocytes and skeletal muscle, but it did not investigate the effects of Cy3G in other tissues. In addition to skeletal muscle and adipose tissue, PGC-1α is expressed in several other tissues, such as the brain and liver, and furthermore has been shown to induce many positive health effects [24]. For example, PGC-1α mediates the adaptation of the liver to nutrient deprivation by inducing gluconeogenesis, fatty-acid β-oxidation, ketogenesis, and bile-acid homeostasis in response to hepatic fasting [137]. Accordingly, PGC-1α deficiency impairs hepatic glucose production and leads to fasting hypoglycemia [137]. Similarly, a lack of PGC-1α expression in the brain is associated

with neuronal diseases and neurodegeneration, for example, depression and Huntington's disease [137]. Thus, whilst this study showed that Cy3G can increase PGC-1α expression in adipocytes and skeletal muscle, further study is needed to investigate whether Cy3G-induced upregulation of PGC-1α may also produce positive effects in other body tissues.

In conclusion, the results presented in this thesis reveal that Cy3G positively regulates metabolic environments by increasing PGC-1α expression via the elevation of intracellular cAMP levels. These effects contribute to both the enhancement of exercise performance and the prevention of metabolic diseases, and indicate that Cy3G may be an effective therapeutic agent for the prevention and/or treatment of lifestyle-related diseases.

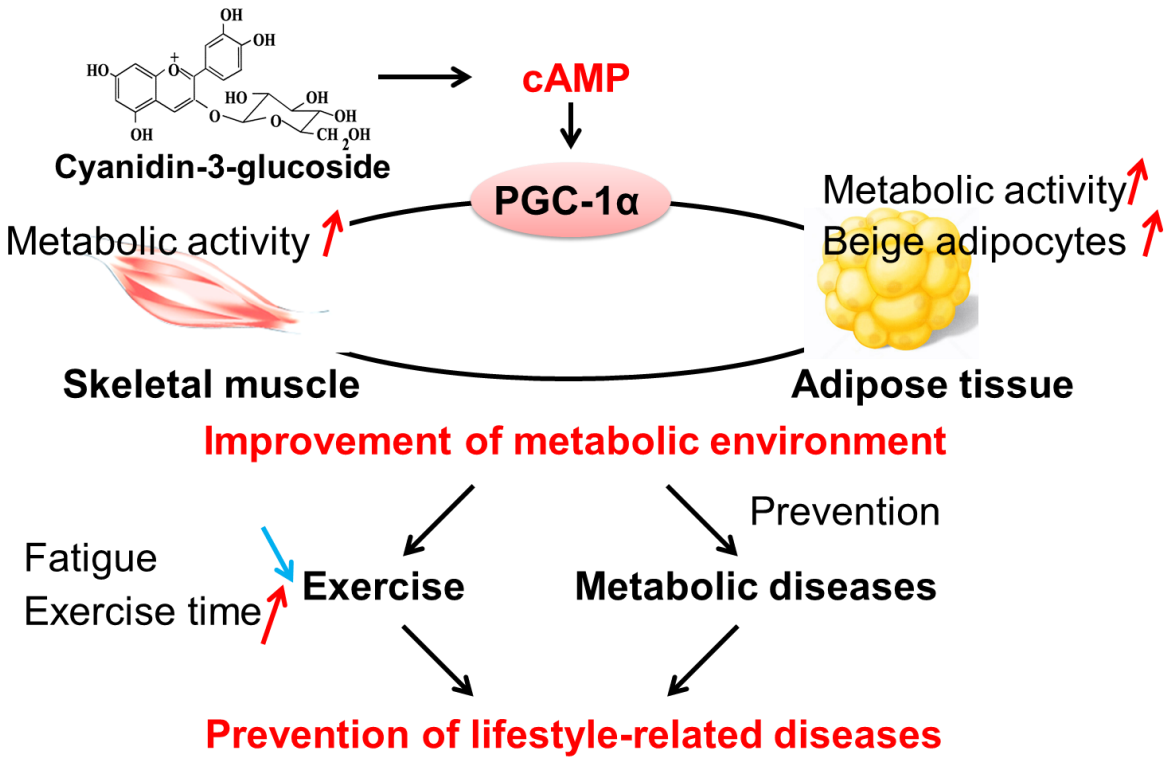


Figure 4-1. The summary of metabolic environment improvement effects of Cy3G. Cy3G positively regulates metabolic environments by increasing PGC-1α expression via the elevation of intracellular cAMP levels. These effects contribute to both the enhancement of exercise performance and the prevention of metabolic diseases, and indicate that Cy3G may be an effective therapeutic agent for the prevention and/or treatment of lifestyle-related diseases.

References

[1] Kopelman PG. Obesity as a medical problem. Nature. 2000; 404: 635-643.

[2] Nursing for the people with lifestyle-related diseases in Japan. Japanese Nursing Association.

[3] Goran M. Energy metabolism and obesity. Medical Clinics of North America. 2000; 84: 347-362.

[4] Hall KD, Heymsfield SB, Kemnitz JW, Klein S, Schoeller DA, Speakman JR. Energy balance and its components: implications for body weight regulation. Am J Clin Nutr. 2012; 95: 989-994

[5] Fukagawa NK, Bandini LG, Young JB. Effect of age on body composition and resting metabolic rate. Am J Physiol. 1990; 259: 233-8.

[6] Zou C, Shao J. Role of adipocytokines in obesity-associated insulin resistance. J Nutr Biochem. 2008; 19: 277-286.

[7] Nasri H, Baradaran A, Shirzad H, Rafieian-Kopaei M. New concepts in nutraceuticals as alternative for pharmaceuticals. Int J Prev Med. 2014; 5, 1487-1499.

[8] González-Castejón M, Rodriguez-Casado A. Dietary phytochemicals and their potential effects on obesity: a review. Pharmacol Res. 2011; 64: 438-455.

[9] Zar Kalai F, Han J, Ksouri R, El Omri A, Abdelly C, Isoda H. Antiobesity Effects of an Edible Halophyte Nitraria retusa Forssk in 3T3-L1 Preadipocyte Differentiation and in C57B6J/L Mice Fed a High Fat Diet-Induced Obesity. Evid Based Complement Alternat Med. 2013; 368658.

[10] Lee J, Jung E, Lee J, Kim S, Huh S, Kim Y, Kim Y, Byun SY, Kim YS, Park D. Isorhamnetin represses adipogenesis in 3T3-L1 cells. Obesity (Silver Spring). 2009; 17: 226-232.

[11] Amie J. Cellular effects of resveratrol in skeletal muscle. Life Sciences. 2009; 84:

637-640.

[12] Nagao T, Meguro S, Soga S, Otsuka A, Tomonobu K, Fumoto S, Chikama A, Mori K, Yuzawa M, Watanabe H, Hase T, Tanaka Y, Tokimitsu I, Shimasaki H, Itakura H. Tea Catechins Suppress Accumulation of Body Fat in Humans. J Oleo Sci. 2001; 50: 717-728

[13] Harada U, Chikama A, Saito S, Takase H, Nagao T, Hase T, Tokumitsu I. Effects of the Long-Term Ingestion of Tea Catechins on Energy Expenditure and Dietary Fat Oxidation in Healthy Subjects. Journal of Health Science. 2005; 51: 248-252.

[14] Ota N, Soga S, Murase T, Shimotoyodome A, Hase T. Consumption of Coffee Polyphenols Increases Fat Utilization in Human. Journal of Health Science. 2010; 56: 745-751.

[15] Ringholm S, Olesen J, Pedersen JT, Brandt CT, Halling JF, Hellsten Y, Prats C, Pileqaard H. Effect of lifelong resveratrol supplementation and exercise training on skeletal muscle oxidative capacity in aging mice; impact of PGC-1α. Exp Gerontol. 2013; 48: 1311-1318.

[16] Kühnau J. The flavonoids. A class of semi-essential food components: their role in nutrition. World Rev Nutr Diet. 1976; 24: 117-191.

[17] Parkinson TM, Brown JP. Metabolic fate of food colorants. Annu. Rev. Nutr. 1981; 1: 175-205.

[18] Chen PN, Chu SC, Chiou HL, Chiang CL, Yang SF, Hsieh YS. Cyanidin 3-glucoside and peonidin 3-glucoside inhibit tumor cell growth and induce apoptosis in vitro and suppress tumor growth in vivo. Nutr Cancer. 2005; 53: 232-243.

[19] Hana J, Kwak HK, Kwang KT. Antioxidant and antiinflammatory activities of cyanidin-3-glucoside and cyanidin-3-rutinoside in hydrogen peroxide and lipopolysaccharide-treated RAW264.7 cells. Food science and Biotechnology. 2014; 23: 2053-2062.

[20] Slavin M, Lu Y, Kaplan N, Yu LL. Effects of baking on cyanidin-3-glucoside content and antioxidant properties of black and yellow soybean crackers**.** Food Chem. 2013; 141:

1166-1174.

[21] Cao G, Muccitelli HU, Sánchez-Moreno C, Prior RL. Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study. Am J Clin Nutr. 2001; 73: 920-6.

[22] Felgines C, Texier O, Garcin P, Besson C, Lamaison JL, Scalbert A. Tissue distribution of anthocyanins in rats fed a blackberry anthocyanin-enriched diet. Mol Nutr Food Res 2009; 53: 1098-1103.

[23] Matsukawa T. Skeletal Muscle Metabolism Regulation Effect of Cyanidin-3-glucoside Derived from Black Soybean (*Glycine max* (L.) Merr). Master Thesis 2015, University of Tsukuba.

[24] Liang H, Ward WF. PGC-1alpha: a key regulator of energy metabolism. Adv Physiol Educ. 2006; 30: 145-151.

[25] Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM. Mechanisms Controlling mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1. Cell. 1999; 98: 115-124.

[26] Boss O, Hagen T, Lowell BB. Uncoupling proteins 2 and 3: potential regulators of mitochondrial energy metabolism. Diabetes. 2000; 49: 143-156.

[27] Zhang L, Keung W, Samokhvalov V, Wang W, Lopaschuk GD. Role of fatty acid uptake and fatty acid β-oxidation in mediating insulin resistance in heart and skeletal muscle. Biochim Biophys Acta. 2010; 1801: 1-22.

[28] Zhou M, Lin BZ, Coughlin S, Vallega G, Pilch PF. UCP-3 expression in skeletal muscle: effects of exercise, hypoxia, and AMP-activated protein kinase. Am J Physiol Endocrinol Metab. 2000; 279: E622-629

[29] Tadaishi M, Miura S, Kai Y, Kano Y, Oishi Y, Ezaki O. Skeletal muscle-specific expression of PGC-1α-b, an exercise-responsive isoform, increases exercise capacity and peak oxygen uptake. PloS One. 2011; 6: e28290.

[30] Handschin C, Choi CA, Chin S, Kim S, Kawamori D, Kupad AJ, Neubauer N, Hu J, Mootha VK, Kim YB, Kulkarni RN, Shulman GI, Spiegelman BM. Abnormal glucose homeostasis in skeletal muscle-specific PGC-1alpha knockout mice. J Clin Invest. 2007; 117: 3463-3474.

[31] Chan MC, Arany Z. The many roles of PGC-1α in muscle-recent developments. Metabolism. 2014; 63: 441-451.

[32] Egan B, Zierath JR. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. Cell Metab. 2013; 17: 162-184.

[33] Lafontan M. Adipose tissue and adipocyte dysregulation. Diabetes Metab. 2014; 40: 16-28.

[34] Rosen ED, Spieqelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. Nature. 2006; 444: 847-853.

[35] Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. Nat Med. 2013; 19: 1252-1263.

[36] Sidossis L, Kajimura S. Brown and beige fat in human: thermogenic adipocytes that control energy and glucose homeostasis. J Clin Invest. 2015; 125: 478-486.

[37] Giralt M, Villarroya F. White, brown, beige/brite: different adipose cells for different functions? Endocrinology. 2013; 154: 2992-3000.

[38] Zurlo F Larson K, Bogardus C, Ravussin E. Skeletal muscle metabolism is a major determinant of resting energy expenditure. The Journal of Clinical Investigation. 1990; 86: 1423-1427.

[39] Carbone JW, McClung JP, Pasiakos SM. Skeletal muscle responses to negative energy balance: effects of dietary protein. Advances in Nutrition. 2012; 3: 119-126.

[40] Safdar A, Little JP, Stokl AJ, Hettinga BP, Akhtar M, Tarnopolsky MA. Exercise increases mitochondrial PGC-1α content and promote nuclear-mitochondrial cross-talk

to coordinate mitochondrial biogenesis. J Biol Chem. 2011; 286, 10605-10617.

[41] Brønnum-Hansen H, Juel K, Davidsen M, Sørensen J. Impact of selected risk factors on quality-adjusted life expectancy in Denmark. Scand. J Public Health. 2007; 35: 510-515.

[42] Yan Z, Okutsu M, Akhtar YN, Lira VA. Regulation of exercise-induced fiber type transformation, mitochondrial biogenesis, and angiogenesis in skeletal muscle. J Appl Physiol (1985). 2011; 110: 264-274.

[43] Coll T, Jove M, Rodriquez-Calvo R, Eyre E, Palomer X, Sanchez RM, Merlos M, Laguna JC, Vázquez-Carrera M. Palmitate-Mediated Downregulation of Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α in Skeletal Muscle Cells Involves MEK1/2 and Nuclear Factor-κB Activation. Diabetes. 2006; 55: 2779-2787.

[44] Verma MK, Yateesh AN, Smitha R, Neelima K, Pallavi PM, Reddy M, Poornima J, Oommen AM, Jagannath MR, Somesh BP. Integrated analysis of chronic lipotoxicity on muscle metabolism and stress and its reversal by antioxidant. Springerplus. 2014; 3: 251.

[45] Nieman DC, Williams AS, Shanely RA, Jin F, McAnulty SR, Triplett NT, Austin MD, Henson DA. Quercetin's influence on exercise performance and muscle mitochondrial biogenesis. Med Sci Sports Exerc. 2010; 42: 338-345.

[46] Burattini S, Ferri P, Battistelli M, Curci R, Luchetti F, Falcieri E. C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. Eur J Histochem. 2004; 48: 223-234.

[47] Johnson LV, Walsh ML, Chen LB. Localization of mitochondria in living cells with rhodamine 123. Proceedings of the National Academy of Sciences of the United States of America. 1980; 77: 990-994

[48] Takeda K, Machida M, Kohara A, Omi N, Takemasa T. Effect of citrulline supplementation on fatigue and exercise performance. J Nutr Sci Vitamino (Tokyo). 2011; 57: 246-250.

[49] Aldeam F, Irving R, Dilworth L. Overview of Lactate Metabolism and the

Implications for Athletes. AJSSM. 2013; 1: 42-46.

[50] Stallknecht B, Vissing J, Galbo H. Lactate production and clearance in exercise. Effects of training. A mini-review. Scand. J Med Sci Sports. 1988; 8: 127-131.

[51] Summermatter S, Santos G, Pérez-Schindler J, Handschin C. Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A. Proc Natl Acad Sci U S A. 2013; 110: 8738-8743.

[52] Hashimoto T, Hussien R, Oommen S, Gohil K, Brooks GA. Lactate sensitive transcription factor network in L6 cells: activation of MCT1 and mitochondrial biogenesis. FASEB J. 2007; 21: 2602-2612.

[53] WillKomm L, Schubert S, Jung R, Elsen M, Borde J, Gehlert S, Suhr F, Bloch W. Lactate regulates myogenesis in C2C12 myoblastes in vitro. Stem cell Res. 2014; 12: 742-753.

[54] Nozaki S, Tanaka M, Mizuno K, Ataka S, Mizuma H, Tahara T, Sugino T, Shirai T, Eguchi A, Okuyama K, Yoshida K, Kajimoto Y, Kuratsune H, Kajimoto O, Watanabe Y. Mental and physical fatigue-related biochemical alterations. Nutrition. 2009; 25: 51-57.

[55] Ehrenborg E, Krook A. Regulation of skeletal muscle physiology and metabolism by peroxisome proliferator-activated receptor δ. Pharmacol Rev. 2009; 61: 373-393.

[56] Jäger S, Handschin C, St-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. Proc Natl Acad Sci U S A. 2007; 104: 12017-12022.

[57] Hardie DG, Hawley SA, Scott JW. AMP-activated protein kinase-development of the energy sensor concept. J Physiol. 2006; 574: 7-15.

[58] Tokumitsu H, Inuzuka H, Ishikawa Y, Ikeda M, Saji l, Kobayashi R. STO-609, a specific inhibitor of the Ca^{2+}/calmodulin-dependent protein kinase kinase. J Biol Chem. 2002; 277: 15813-15818.

[59] Jensen TE, Woitaszewski JF, Richter EA. AMP-activated protein kinase in contraction regulation of skeletal muscle metabolism: necessary and/or sufficient?. Acta Physiologica. 2009; 196: 155-174.

[60] Wehrrens XH, Lehnart SE, Reiken SR, Marks AR. Ca^{2+}/calmodulin-dependent protein kinase kinase II phosphorylation regulates the cardiac ryanodine receptor. Circ Res. 2004; 94: e61-e70.

[61] Brun JF, Dumortier M, Fedou C, Mercier L. Exercise hypoglycemia in nondiabetic subjects. Diabetes Metab. 2001; 27: 92-106.

[62] Pilegaard H, Saltin B, Neufer PD. Exercise induces transient transcriptional activation of the PGC-1α gene in human skeletal muscle. J Physiol. 2003; 546: 851-858.

[63] Mathai AS, Bonen A, Benton CR, Robinson DL, Graham TE. Rapid exercise-induced changes in PGC-1alpha mRNA and protein in human skeletal muscle. J Appl Physiol (1985). 2008; 105: 1098-1105.

[64] Halestrap AP, Meredith D. The SLC16 gene family from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pfluqers Arch. 2004; 447: 619-628.

[65] Svensson K, Albert V, Cardel B, Salatino S, Handschin C. Skeletal muscle PGC-1α modulates systemic ketone body homeostasis and ameliorates diabetic hyperketonemia in mice. FASEB J. 2016; 30: 1976-1986.

[66] Schreiber SN, Emter R, Hock MB, Knutti D, Cardenas J, Podvinec M, Oakeley EJ, Kralli A. The estrogen-related receptor α (ERRα) functions in PPARγ coactivator1α (PGC-1α)-induced mitochondrial biogenesis. Proc Natl Acad Sci U S A. 2004; 101: 6472-6477.

[67] Chinsomboon J, Ruas J, Gupta RK, Thom R, Shoag J, Rowe GC, Sawada N, Raghuram S, Arany Z. The transcriptional coactivator PGC-1α mediates exercise-induced angiogenesis in skeletal muscle. Proc Natl Acad Sci U S A. 2009; 106, 21401-21406.

[68] Delavar H, Noqueira L, Waqner PD, Hoqan MC, Metzqer D, Breen EC. Skeletal

myofiber VEGF is essential for the exercise training response in adult mice. Am J Physiol Requl Inteqr Comp Physiol. 2014; 306, 586-595.

[69] Fogarty S, Hawley SA, Green KA, Saner N, Mustard KJ, Hardie DG. Calmodulin-dependent protein kinase kinase-β activates AMPK without forming a stable complex: synergistic effects of Ca^{2+} and AMP. Biochem J. 2010; 426: 109-118.

[70] Handschin C, Rhee J, Lin J, Tarr PT, Spiegelman BM. An autoregulatory loop controls peroxisome proliferator-activated receptor γ coactivator 1α expression in muscle. Proc Natl Acad Sci U S A. 2003; 100: 7111-71116.

[71] Zhu W, Jia Q, Wang Y, Zhang Y, Xia M. The anthocyanin cyanidin-3-O-β-glucoside, a flavonoid, increases hepatic glutathione synthesis and protects hepatocytes against reactive oxygen species during hyperglycemia: Involvement of a cAMP-PKA-dependent signaling pathway. Free Radic Biol Med. 2012; 52: 314-327.

[72] Omar B, Zmuda-Trzebiatowska E, Manganiello V, Göransson O, Degerman E. Regulation of AMP-activated protein kinase by cAMP in adipocytes: roles for phosphodiesterases, protein kinase B, protein kinase A, Epac and lipolysis. Cell Signal. 2009; 21, 760-766.

[73] Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. J Clin Invest. 2006; 116: 615-622.

[74] Bender AT, Beavo JA. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol Rev. 2006; 58: 488-520.

[75] Dallas C, Gerbi A, Tenca G, Juchaux F, Bernard FX. Lipolytic effect of a polyphenolic citrus dry extract of grapefruit, orange (SINETROL) in human body fat adipocytes. Mechanism of action by inhibition of cAMP-phosphodiesterase (PDE). Phytomedicine. 2008; 15: 783-792.

[76] Kelley DE, He J, Menshikova EV, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes. 2002; 51:2944-50.

[77] Merlin J, Evans BA, Dehvari N, Sato M, Bengtsson T, Hutchinson DS. Could

burning fat start with a brite spark? Pharmacological and nutritional ways to promote thermogenesis. Mol Nutr Food Red. 2016; 60: 18-42.

[78] Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol. 2008; 9: 367-377.

[79] Zhuo X, Zhang P, Hoerger TJ. Lifetime Direct Medical Costs of Treating Type 2 Diabetes and Diabetic Complications. Am J Prev Med. 2013; 45: 253-261.

[80] Nawrocki AR, Scherer PE. Keynote review: The adipocytes as a drug discovery target. Drug Discov Today. 2005; 10: 1219-1230.

[81] Mueller E. Understanding the variegation of fat: novel regulators of adipocyte differentiation and fat tissue biology. Biochim Biophys Acta. 2014; 1842: 352-357.

[82] Flachs P, Rossmeisl M, Kuda O, Kopecky J. Stimulation of mitochondrial oxidative capacity in white fat independent of UCP1: A key to lean phenotype. Biochim Biophys Acta. 2013; 1831: 986-1003.

[83] Bonet ML, Oliver P, Palou A. Pharmacological and nutritional agents promoting browning of white adipose tissue. Biochim Biophys Acta. 2013; 1831: 969-985.

[84] Ohno H, Shinoda K, Spieqelman BM, Kajimura S. PPARγ agonists Induces a White-to-Brown Fat Conversion through Stabilization of PRDM16 Protein. Cell Metab. 2012; 15: 395-404.

[85] Fu J, Li X, Zhang Y, Mao Y, Wang A, Wang X, Zou Z, Zhang X. Molecular pathways regulating the formation of brown-like adipocytes in white adipose tissue. Diabetes Metab Res Rev. 2015; 31: 433-52.

[86] Karamanlidis G, Karamitri A, Docherty K, Hazlerigg DG, Lomax MA. C/EBPβ reprograms white 3T3-L1 preadipocytes to a brown adipocyte pattern of gene expression. J Biol Chem. 2007; 282. 24660-24669.

[87] Chi J, Cohen P. The multifaceted roles of PRDM16: Adipose Biology and beyond.

Trends Endocrinol Metab. 2016; 27: 11-23.

[88] Kanamoto Y, Yamashita Y, Nanba F, Yoshida T, Tsuda T, Fukuda I, Nakamura-Tsuruta S, Ashida H. A black soybean seed coat extract prevents obesity and glucose intolerance by up-regulating uncoupling proteins and down-regulating inflammatory cytokines in high-fat diet-fed mice. Journal of Agricultural and Food Chemistry. 2011; 59: 8985-8993.

[89] Lee JH, Kang NS, Shin SO, Shin SH, Lim SG, Suh DY *et al*. Characterisation of anthocyanins in the black soybean (*Glycine max* L.) by HPLC-DAD-ESI/MS analysis. Food Chem. 2009; 112: 226-231.

[90] Choung MG, Baek IY, Kang ST, Han WY, Shin DC, Moon HP *et al*. Isolation and determination of anthocyanins in seed coats of black soybean (Glycine max (L.) Merr.). J Agric Food Chem. 2001; 49: 5848-5855.

[91] Kurimoto Y, Shibayama Y, Inoue S, Soga M, Takikawa M, Ito C, Nanba F, Yoshida T, Yamashita Y, Ashida H, Tsuda T. Black soybean seed coat extract ameliorates hyperglycemia and insulin sensitivity via the activation of AMP-activated protein kinase in diabetic mice. J Agric Food Chem. 2013; 61: 5558-5564.

[92] Green H, Kehinde O. An established preadipose cell line and its differentiation in culture II. Factors affecting the adipose conversion. Cell. 1975; 5: 19-27.

[93] Cristacho AG, Lazar MA. Forming function fat: a growing understanding of adipocyte differentiation. Nat Rev Mol Cell Biol. 2011; 12: 722-734.

[94] Iwabu M, Yamauchi T, Okada-Iwabu M, Sato K, Nakagawa T, Funata M, Yamaguchi M, Namiki S, Nakayama R, Tabata M, Ogata H, Kubota N, Takamoto I, Hayashi YK, Yamauchi N, Waki H, Fukayama M, Nishino I, Tokuyama K, Ueki K, Oike Y, Ishii S, Hirose K, Shimizu T, Touhara K, Kadowaki T. Adiponectin and AdipoR1 regulate PGC-1alpha and mitochondria by Ca(2+) and AMPK/SIRT1. Nature. 2010; 464: 1313-9.

[95] Brondani LA, Assmann TS, Duarte GC, Gross JL, Canani LH, Crispim D. The role of the uncoupling protein 1 (UCP1) on the development of obesity and type 2 diabetes

mellitus. Arg Bras Endocrinol Metabol. 2012; 56: 215-225.

[96] Pardo R, Enquix N, Lasheras J, Feliu JE, Kralli A, Villena JA. Rosiglitazone-Induced Mitochondrial Biogenesis in White Adipose Tissue Is Independent of Peroxisome Proliferator-Activated Receptor γ Coactivator-1α. PloS One. 2011; 6: e26989.

[97] Butow RA, Bahassi EM. Adaptive thermogenesis: Orchestrating mitochondrial biogenesis. Curr Biol. 1999; 9: 767-769.

[98] Sharp LZ, Shinoda K, Ohno H, Scheel DW, Tomoda E, Ruiz L, Hu H, Wang L, Pavlova Z, Gilsanz V, Kajimura S. Human BAT possesses molecular signatures that resemble beige/brite cells. PLoS One. 2012; 7: e49452.

[99] Farmer SR. Transcriptional control of adipocyte formation. Cell Metab. 2006; 4: 263-273.

[100] Darlington GJ, Ross SE, MacDougald OA. The role of C/EBP Genes in Adipocyte Differentiation. J Biol Chem. 1998; 273: 30057-30060.

[101] Siersbaek R, Nielsen R, Mandrup S. Transcriptional networks and chromatin remodeling controlling adipogenesis. Trends Endocrinol Metab. 2012; 23: 56-64.

[102] Tartaglia LA. The Leptin Receptor. J Biol Chem. 1997; 272: 6093-6096.

[103] Hummel KP, Dickie MM, Coleman DL. Diabetes, a New Mutation in the Mouse. Science. 1966; 153: 1127-1128.

[104] Badshah H, Ullah I, Kim SE, Kim TH, Lee HY, Kim MO. Anthocyanins attenuate body weight gain via modulating neuropeptide Y and $GABA_{B1}$ receptor in rats hypothalamus. Neuropeptides. 2013; 47: 347-353.

[105] Sivitz WI, Yorek MA. Mitochondrial Dysfunction in Diabetes: From Molecular Mechanisms to Functional Significance and Therapeutic Opportunities. Antioxid Redox Signal. 2010; 12: 537-577.

[106] Chevilloate E, Giralt M, Miroux B, Ricquier D, Villarroya F. Uncoupling protein-

2 controls adiponectin gene expression in adipose tissue through the modulation of reactive oxygen species production. Diabetes. 2007; 56: 1042-1050.

[107] Tiganis T. Reactive oxygen species and insulin resistance: the good, the bad and the ugly. Trends Pharmacol Sci. 2011; 32: 82-93.

[108] Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol. 2012; 13: 251-262.

[109] Daval M, Foufelle F, Ferré P. Functions of AMP-activated protein kinase in adipose tissue. J Physiol. 2006; 574: 55-62.

[110] De Pauw A, Tejerina S, Raes M, Keijer J, Arnould T. Mitochondrial (Dys) function in Adipocyte (De) differentiation and Systemic Metabolic Alteration. Am J Pathol. 2009; 175: 927-939.

[111] Lu R, Ji H, Chang ZG, Su SS, Yang GS. Mitochondrial development and the influence of its dysfunction during rat adipocytes differentiation. Mol Biol Rep. 2010; 37: 2173-2182.

[112] Asano H, Kanamori Y, Higurashi S, Nara T, Kato K, Matsui T, Funada M. Induction of beige-like adipocytes in 3T3-L1 adipocytes. J Vet Med Sci. 2014; 76: 57-64.

[113] Stine RR, Shapira SN, Lim HW, Ishibashi J, Harms M, Won KJ, Seale P. EBF2 promotes the recruitment of beige adipocytes in white adipose tissue. Mol Metab. 2015; 5: 57-65.

[114] Hassan M, El Yazidi C, Landrier JF, Lairon D, Margotat A, Amiot MJ. Phloretin enhances adipocyte differentiation and adiponectin expression in 3T3-L1 cells. Biochem Biophys Res Commun. 2007; 361: 208-123.

[115] Saito T, Abe D, Sekiya K. Flavanone exhibits PPARγ ligand activity and enhances differentiation of 3T3-L1 adipocytes. Biochem Biophys Res Commun. 2009; 380: 281-285.

[116] Saito T, Abe D, Sekiya K. Nobiletin enhances differentiation and lipolysis of 3T3-

L1 adipocytes. Biochem Biophys Res Commun. 2007; 357: 371-376.

[117] Saito T, Abe D, Sekiya K. Sakuranetin induces adipogenesis of 3T3-L1 cells through enhanced expression of PPARgamma2. Biochem Biophys Res Commun. 2008; 372: 835-839.

[118] Wu Z, Rosen DE, Brun R, Hauser S, Adelmant G, Troy AE, McKeon C, Darlington GJ, Spiegelman BM. Cross-regulation of C/EBPα and PPARγ controls the transcriptional pathway of adipogenesis and insulin sensitivity. Mol Cell. 1999; 3: 151-158.

[119] Rosen EC, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ, Spieqelman BM. C/EBPα induces adipogenesis through PPARγ: a unified pathway. Genes Dev. 2002; 16: 22–6.

[120] Mineo H, Oda C, Chiji H, Kawada T, Shimizu K, Taira T. Thiazolidinediones exhibit different effects on preadipocytes isolated from rat mesenteric fat tissue and cell line 3T3-L1 cells derived from mice. Cell Biol Int. 2007; 31: 703-710.

[121] Houseknecht KL, Cole BM, Steele PJ. Peroxisome proliferator-activated receptor gamma (PPARγ) and its ligands: A review. Domes Anim Endocrinol. 2002; 22: 1-23.

[122] Karnieli E, Armoni M. Transcriptional regulation of the insulin-responsive glucose transporter GLUT4 gene: from physiology to pathology. Am J Physiol Endocrinol Metab. 2008; 295: 38-45.

[123] Yand D, Guo J, Divieti P, Shioda T, Bringhurst FR. CBP/p300-Interacting Protein CITED1 Modulates Parathyroid Hormone Regulation of Osteoblastic Differentiation. Endocrinology. 2008; 149: 1728-1735.

[124] Fu Y, Li F, Zhao DY, Zhang JS, Lv Y, Li-Ling J. Interaction between Tbx1 and HoxD10 and connection with TGFβ-BMP signal pathway during kidney development. Gene. 2014; 536: 197-202.

[125] Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. J Agric Food Chem. 2006; 54: 4069-4075.

[126] Pedersen BK. Muscle as a Secretory Organ. Comprehensive Physiology. 2013; 3: 1337-1361.

[127] Yamauchi T, Kamon J, Waki H, Murakami K, Motojima K, Komeda K, Ide T, Kubota N, Terauchi Y, Tobe K, Miki H, Tsuchida A, Akanuma Y, Nagai R, Kimura S, Kadowaki T. The mechanisms by which both heterozygous peroxisome proliferator-activated receptor gamma (PPARgamma) deficiency and PPARgamma agonist improve insulin resistance. J Biol Chem. 2001; 276: 41245-41254.

[128] Smith SA. Central role of the adipocyte in the insulin-sensitising and cardiovascular risk modifying actions of the thiazolidinediones. Biochimie. 2003; 85: 1219-1230.

[129] Miyazawa T, Nakagawa K, Kudo M, Muraishi K. Direct Intestinal Absorption of Red Fruit Anthocyanins, Cyanidin-3-glucoside and Cyanidin-3,5-diglucoside, into Rats and Humans. J Agric Food Chem. 1999; 47: 1083-1091.

[130] Tsuda T, Ueno Y, Kojo H, Yoshikawa T, Osawa T. Gene expression profile of isolated rat adipocytes treated with anthocyanins. Biochim Biophys Acta. 2005; 1733: 137-147.

[131] He YH, Xiao C, Wang YS, Zhao LH, Zhao HY, Tong Y, Zhou J, Jia HW, Lu C, Li XM, Lu AP. Antioxidant and anti-inflammatory effects of cyanidin from cherries on rat adjuvant-induced arthritis. Zhongguo Zhong Yao Za Zhi. 2005; 30: 1602-1605.

[132] Zou TB, Feng D, Song G, Li HW, Tang HW, Ling WH. The role of sodium-dependent glucose transporter 1 and glucose transporter 2 in the absorption of cyanidin-3-o-β-glucoside in Caco-2 cells. Nutients. 2014; 6: 4165-4177.

[133] Peluso MR. Flavonoids attenuate cardiovascular disease, inhibit phosphodiesterase, and modulate lipid homeostasis in adipose tissue and liver. Exp Biol Med (Maywood). 2006; 231: 1287-1299.

[134] Beretz A, Anton R, Stoclet CJ. Flavonoid compounds are potent inhibitors of cyclic AMP phosphodiesterase. Experientia. 1978; 8: 1054-1055.

[135] Francis SH, Blount MA, Corbin JD. Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions. Physiol Rev. 2011; 91: 651-690.

[136] Iwashita K, Yamaki K, Tsushida T. Effect of flavonoids on the differentiation of 3T3-L1 adipocytes. Food Sci Technol Res. 2001; 7: 154-160.

[137] Lin J, Handschin C, Spiegelman BM. Metabolic control through the PGC-1 family of transcription coactivators. Cell Metab. 2005; 1: 361-370.

ACKNOWLEDGEMENT

I would like to express my sincerest gratitude to many people who gave me instructions, their cooperation, and support during the course of this study:

First of all, I would like to express my profound gratitude to my Ph.D. supervisor, Professor Hiroko Isoda (University of Tsukuba) for accepting me in her laboratory and for giving me help, support, advise and guidance;

I would like to express my profound gratitude Assistant Professor Myra O Villareal (University of Tsukuba), Zeng Wang (University of Tsukuba), and Shinya Takahashi (University of Tsukuba) for giving their support and advises;

I appreciate Professor Zhenya Zhang (University of Tsukuba), Associate Professor Yusaku Miyamae (University of Tsukuba), and Assistant Professor Yasuhiro Shinkai (University of Tsukuba), member of committee Doctor thesis evaluation, for the comments on my thesis and for helping me understand and enrich my ideas;

I appreciate Professor Shinobu Sato (University of Tsukuba) and Associate Professor Keiko Yamaji (University of Tsukuba), member of committee Special Exercises of Sustainable Environmental Studies, for the comments on my thesis and for helping me understand and enrich my ideas;

I would like to express my profound gratitude Professor Masaya Nagao (Graduate School of Biostudies, Kyoto University) for help in the measurement of PPARγ agonist activity of cyanidin-3-glucoside samples.

I would like to express my sincere thanks to the members of Prof. Isoda's laboratory for their support and help: Dr. Junkyu Han, Dr. Feten Zar Kalai, Dr. Shinji Kondo, Dr. Hideko Motojima, Dr. Hiroyuki Kozu, Mr. Tetsuya Inaguma, Ms. Ayumi Ikeya, Ms. Sayo Arao, Ms. Yuki Sato, Ms. Akiko Jitsuhiro, Ms. Midori Sakamaki, Ms. Yukino Hataoka, and Ms. Natsumi Inoue.

Finally, I would like to express my profound gratitude to my parents for their encouragement and support throughout the years.