Dual roles of benzyl isothiocyanate in the glucose uptake and lipid accumulation in adipocytes

September 2018

Ying Liang

Graduate School of Environmental and Life Science

(Doctor's Course)

Okayama University, Japan

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ABBREVIATIONS

BITC, benzyl isothiocyanate

AITC, allyl isothiocyanate

ITCs, isothiocyanates

IBMX, 3-isobutyl-1-methylxanthine

DEX, dexamethasone

FBS, fetal bovine serum

CS, calf serum

PPAR-γ, peroxisome proliferator-activated receptor-gamma

C/EBPs, CCAAT/enhancer-binding proteins

GLUT4, glucose transporter 4

AMPK, 5′-AMP-activated protein kinase

MAPKs, mitogen activated protein kinases

ERK, extracellular signal-regulated kinase

JNK, c-Jun N-terminal kinase

PI3K, phosphoinositide 3-kinase

Akt, protein kinase B

Comp. C, compound C

2DG, 2-deoxyglucose

DG6P, deoxyglucose-6-phosphate

TNF-a, tumor necrosis factor alpha

MCE, mitotic clonal expansion

ABSTRACT

Obesity and obesity-induced insulin resistance increase the risk of the major diseases closely related to metabolic syndrome, such as heart disease, stroke, hyperglycemia and type 2 diabetes. Adipose tissue, a highly insulin-responsive tissue, plays an important role in the regulation of glucose and lipid metabolism, insulin action, energy balance and inflammation. Adipocyte differentiation involves a series of transcriptional activators, including peroxisome proliferator-activated receptor-gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBPs). Glucose transporter 4 (GLUT4), also regulated by cooperation of PPARγ and C/EBPα, plays a pivotal role in glucose uptake in adipocytes. In addition, insulin resistance is characterized by the reduced glucose uptake by the target cells owing to impaired insulin signaling transduction and/or glucose transporting ability. Besides, enlarged adipocytes secret several proinflammatory factors, such as tumor necrosis factor-alpha (TNF-α), which lead to insulin resistance.

Benzyl isothiocyanate (BITC), an aromatic isothiocyanate compound derived from papaya, has been demonstrated to restrain the high-fat diet-induced body weight gain and liver fat accumulation. However, it remains unclear whether BITC can influence the glucose uptake and lipid accumulation in adipocytes. In this study, I have focused on the molecular mechanisms involved in the glucose and lipid metabolism influenced by BITC.

In the Chapter 2, the inhibitory effects of BITC on lipid accumulation in 3T3-L1 preadipocytes during differentiation-inducing was investigated. The treatment of BITC during the differentiation-inducing stage significantly ameliorated the lipid accumulation, whereas it had no inhibitory effect during the differentiation-maintaining stage. BITC also significantly suppressed the mRNA expression of the adipocyte-specific markers, such as C/EBPα, C/EBPβ, C/EBPδ and PPARγ. BITC significantly

inhibited the phosphorylation of ERK1/2 phosphorylation, whereas it enhanced that of 5′-AMP-activated protein kinase (AMPK). Furthermore, BITC significantly suppressed the intracellular 2-deoxyglucose uptake as well as GLUT4 gene expression. These results suggest that inhibition of the adipocyte differentiation and glucose uptake may mainly contribute to the inhibitory effect of BITC on the lipid accumulation in 3T3-L1 preadipocytes.

In the Chapter 3, I investigated whether BITC can ameliorate insulin resistance in 3T3-L1 mature adipocytes. The treatment of BITC significantly enhanced insulin-induced glucose uptake in the mature adipocytes. BITC also activated both phosphoinositide 3-kinase (PI3K)/Akt signaling pathway and mitogen activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) in the mature adipocytes. A PI3K inhibitor, LY294002, significantly attenuated the BITC-enhanced glucose uptake and lipid accumulation, whereas PD98059, an ERK signaling inhibitor, did not. Furthermore, BITC actually ameliorated the glucose uptake in the TNF-α-induced insulin resistance in the mature adipocytes. These results suggested that BITC can improve the insulin resistance through the enhanced activation of the PI3K/Akt signaling pathway.

The present study provides biological evidence that (1) BITC inhibits lipid accumulation and glucose uptake in 3T3-L1 preadipocytes, possibly through inhibition of the adipocyte differentiation with the decreased expression of the adipogenic genes; (2) BITC improves the insulin resistance in the mature 3T3-L1 adipocytes, possibly through the enhanced glucose uptake with the activated PI3K/Akt pathway. In conclusion, the present study indicated that BITC is one of the potential food phytochemicals for prevention against not only obesity, but also obesity-related chronic diseases, such as insulin resistance-induced type 2 diabetes.

CHAPTER 1

General introduction

1.1 Obesity

Obesity has increased at an alarming rate in recent years and is now a worldwide public health problem. Obesity is defined as an abnormal increase in fat, even without necessary gain in body weight. Obesity is characterized by an increased adipocyte size (hypertrophy) and adipocyte number (hyperplasia). Obesity and obesity-induced insulin resistance increase the risk of the major diseases closely related to metabolic syndrome, such as heart disease, stroke, hyperglycemia and type 2 diabetes. Adipose tissue is a highly insulin-responsive tissue, which plays an important role in the regulation of glucose and lipid metabolism, insulin action, energy balance and inflammation. Thus, weight loss has been recognized as the major health beneficial way for overweight people and also increases life expectancy in people having obesity-related complication.

1.2 Adipocyte differentiation

Adipocyte differentiation is a complex and programmed process in which new adipocytes are derived from multipotent stem cells or preadipocyte precursors (Rosen & Spiegelman, 2000) Briefly, adipocyte differentiation is divided into four steps, including initial growth arrest, mitotic clonal expansion (MCE), early differentiation, and terminal differentiation–development of mature adipocyte phenotype (Rosen et al., 2000; Gregoire et al., 1998). Before differentiation induction, preadipocytes are similar to fibroblasts. After MCE, as lipid droplets accumulate, preadipocytes convert to a spherical shape and progressively acquire the morphological and biochemical characteristics of mature adipocytes (Gregoire et al., 1998).

During the differentiation of preadipocytes into adipocytes, a series of

transcription factors sequentially and coordinately modulate the expression of hundreds of genes responsible for the establishment of the mature fat-cell phenotype (Rosen et al., 2006; Farmer, 2006). Among those transcription factors, CCAAT/enhancer-binding protein β (C/EBPβ), peroxisome proliferator-activated receptor gamma (PPAR-γ) and CCAAT/enhancer-binding protein α (C/EBPα) are the key factors responsible for adipogenesis (Siersbaek et al., 2010). Glucose transporter 4 (GLUT4), whose expression is also regulated by C/EBPs, plays an important role in the glucose uptake by adipocytes (Kaestner et al., 1990). AMP-activated protein kinase (AMPK) inactivates acetyl CoA carboxylase (ACC) and glycerol-3-phosphate acyl transferase-1, both of which play a key role in the lipogenesis of adipocytes (Carling et al., 2011). The activation of AMPK also contributes to the inhibition of adipocyte differentiation through down-regulation of C/EBPs and PPAR-γ (Gao et al., 2008). The extracellular signal-regulated kinase 1/2 (ERK1/2), a mitogen-activated protein kinase (MAPK), is another member of the protein kinases that regulate the adipocyte differentiation (Sale et al., 1995).

3T3-L1 is a widely used preadipocyte cell line for studying the adipocyte differentiation program. The molecular mechanisms underlying differentiation of adipocytes from preadipocytes have been mostly derived from 3T3-L1 cells. Confluent 3T3-L1 preadipocytes can differentiated by an adipogenic cocktail-DMI (Fig.1.1), which indicates dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), and insulin, respectively (Wang & Hai, 2015). DEX, a glucocorticoid, subsequently translocate into the nucleus and regulate the transcription of genes, including C/EBPδ and C/EBPα (Wu et al., 1996; Tontonoz et al., 1994). As a phosphodiesterase (PDE) inhibitor and adenosine receptor antagonist, IBMX can raise cAMP levels, activate PKA, promote CREB phosphorylation, and thus increase the expression of C/EBPβ (Zhang et al., 2004). Insulin, acts as a proadipogenic agonist, interacts with insulin receptor, activates MAPKs and Akt, and increases the expression of PPAR-γ and

C/EBPα (Rosen et al., 2006; Oberbauer, 2014).

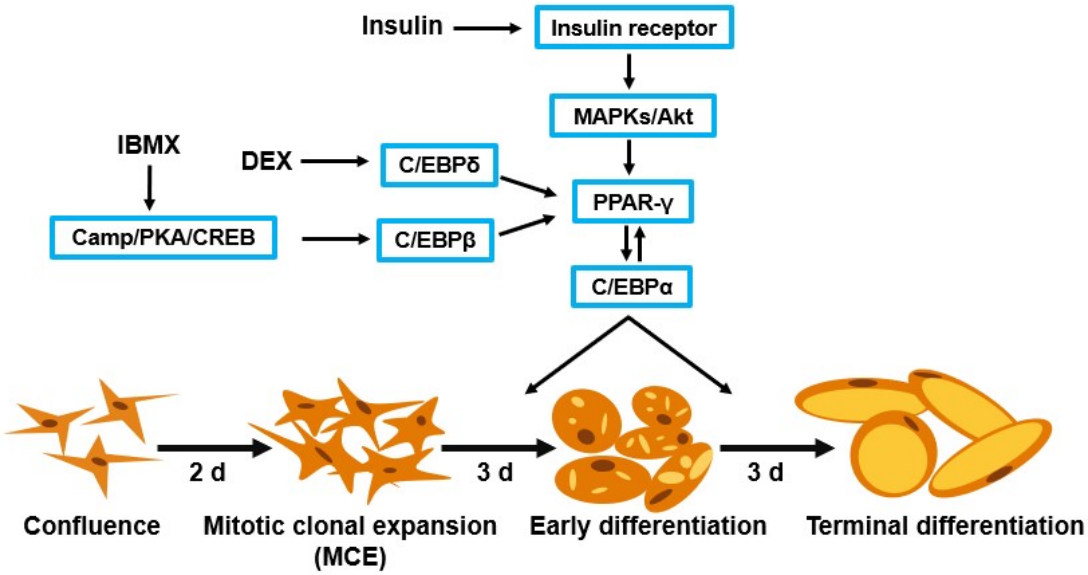


Figure 1.1 Molecular mechanism of DMI-induced adipocyte differentiation.

1.3 Insulin resistance

Insulin resistance is characterized by reduce glucose uptake by target tissue or cell owing to impaired insulin signaling transduction and/or glucose transporting ability. Enlarged adipocytes secret proinflammatory factors, such as tumor necrosis factor-alpha (TNF-α), which lead to insulin resistance. In adipocytes, inactivation of insulin signaling molecules such as insulin receptor, insulin receptor substrate, phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt), especially the down-stream PI3K and Akt, reduces the translocation of GLUT4 and consequently impairs the transporting ability of the cell.

1.4 Benzyl isothiocyanate

Isothiocyanates (ITCs), well-studied electrophilic compounds, are naturally occurring in abundance in cruciferous vegetables including broccoli, watercress,

Brussels sprouts, cabbage, Japanese radish and cauliflower (Nakamura et al., 2018). ITCs are regarded to play significant roles in affording the cancer chemopreventive potentials of these vegetables, because they are capable of up-regulating the xenobiotic-detoxifying enzymes, inducing apoptosis, and inhibiting cell cycle progression (Nakamura et al., 2018; Nakamura et al., 2010). Benzyl isothiocyanate (BITC), an aromatic ITC derived from papaya (Nakamura et al., 2007), has been demonstrated to induce phase 2 drug-metabolizing enzymes (Nakamura et al., 2000) and alcohol-metabolizing enzymes (Liu et al., 2017). BITC also inhibits cell proliferation in various cell lines, such as T lymphocytes (Miyoshi et al., 2004). renal proximal tubular cells (Abe et al., 2012), and colorectal cancer cells (Abe et al., 2014). BITC is also able to inhibit inflammatory responses in macrophages (Murakami et al., 2003) and the cytokine expression (Tang et al., 2015; Tang et al., 2018). In addition, BITC has recently been reported to restrain the high-fat diet-induced body weight gain and liver fat accumulation (Alsanea et al., 2017).

CHAPTER 2

Benzyl isothiocyanate ameliorates lipid accumulation in 3T3-L1 preadipocytes during adipocyte differentiation

2.1 Abstract

Benzyl isothiocyanate (BITC) is an organosulfur compound derived from cruciferous vegetables and papaya seeds. In this study, we investigated the effect of BITC on the lipid accumulation in 3T3-L1 preadipocytes during adipocyte differentiation. The treatment of BITC during the differentiation-inducing stage significantly ameliorated the lipid accumulation, whereas it had no inhibitory effect during the differentiation-maintaining stage. BITC also significantly suppressed the mRNA expression of the adipocyte-specific markers, such as CCAAT/enhancer-binding protein-α (C/EBP-α), C/EBPβ, C/EBP-δ and peroxisome proliferator-activated receptor gamma (PPAR-γ). BITC significantly inhibited the phosphorylation of extracellular signal-regulated kinase phosphorylation, whereas it enhanced that of AMP-activated protein kinase. Furthermore, BITC significantly suppressed the intracellular 2-deoxyglucose uptake as well as glucose transporter 4 expression. These results suggest that inhibition of the adipocyte differentiation and glucose uptake may mainly contribute to the inhibitory effect of BITC on the lipid accumulation in 3T3-L1 preadipocytes.

2.2 Introduction

Adipocytes play an important role in not only maintaining energy homeostasis, but also adipogenesis as a consequence of caloric overabundance (Unger et al., 2010). Adipogenesis is a process of the intracellular accumulation of triacyl glycerol by the differentiation, enlargement with lipogenesis and increase in the number of adipocytes (Spiegelman et al., 1996). Since adipogenesis mainly contributes to the onset and

progression of obesity, the inhibition of adipocyte differentiation, down-regulation of lipogenesis, and enhancement of lipolysis are regarded as promising strategies in preventing the development of obesity (Goto et al., 2015; Wang et al.,2014 Goto et al.,2013).

The differentiation of preadipocytes into adipocytes is regulated by an elaborate transcriptional network such that the coordinated expression of hundreds of proteins is responsible for the maturing adipocytes (Farmer, 2006). Among the transcriptional factors related to the adipocyte differentiation, CCAAT/enhancer-binding protein β (C/EBPβ), peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer-binding protein α (C/EBPα) are master regulators for the formation of mature adipocytes (Siersbaek et al., 2010). Glucose transporter 4 (GLUT4), whose expression is also regulated by C/EBPs, plays an important role in the glucose uptake by adipocytes (Kaestner et al., 1990). AMP-activated protein kinase (AMPK) inactivates acetyl CoA carboxylase (ACC) and glycerol-3-phosphate acyl transferase-1, both of which play a key role in the lipogenesis of adipocytes (Carling et al., 2011). The activation of AMPK also contributes to the inhibition of adipocyte differentiation through down-regulation of C/EBPs and PPAR-γ (Gao et al., 2008). The extracellular signal-regulated kinase 1/2 (ERK1/2), a mitogen-activated protein kinase (MAPK), is another member of the protein kinases that regulate the adipocyte differentiation (Sale et al., 1995).

In the present study, we assessed BITC as a potential inhibitor of lipid accumulation in preadipocytes using the 3T3-L1 mouse fibroblast cell line, a well-characterized cell culture model for the analysis of adipocyte-specific differentiation (Green & Kehinde, 1975). We also investigated the stage-specific effect of BITC on the lipid accumulation and the related signaling molecules, such as transcriptional factors, protein kinases and glucose transporter. The present results suggest that the

inhibition of adipocyte differentiation and glucose uptake might mainly contribute to the inhibitory effect of BITC on the lipid accumulation in 3T3-L1 preadipocytes.

2.3 Materials and Methods

2.3.1 Materials

BITC were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Antibodies against phosphorylated-ERK, ERK, phosphorylated-AMPK, AMPK, and GAPDH were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase-linked anti-rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fetal bovine serum (FBS) and calf serum (CS) were purchased from Nichirei Corporation (Tokyo, Japan). Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Oil Red O solution was purchased from Cayman Chemical (Ann Arbor, MI, USA). Allyl ITC (AITC) was purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals including Chemi-Lumi One Super, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

2.3.2 Cell culture and treatments

Mouse fibroblast cell line 3T3-L1 cells (ATCC® CL‐173™) were cultured in DMEM (Dulbecco’s modified Eagle’s medium, high glucose) supplemented with 10% calf serum and 1% penicillin/streptomycin at 37°C in a humidified chamber of 95% air and 5% CO_2. After the cells were grown to the contact inhibition stage, differentiation was induced using the differentiation-inducing medium (DMEM supplemented 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 10 μg/mL insulin) for 3 days in the same medium. The cells were then cultured in the differentiation-maintaining medium (DMEM supplemented 10% FBS and 10 μg/mL insulin) for 3 days. Thereafter, the cells were cultured in DMEM supplemented

10% FBS for another 2 days. To investigate whether BITC is capable of inhibiting the intercellular lipid accumulation, the cells were treated with BITC or DMSO alone as a vehicle control at the time of the medium change into the differentiation-inducing medium, differentiation-maintaining medium or both media. To investigate the molecular mechanism by which BITC inhibits the adipocyte differentiation, the cells were treated with BITC or DMSO alone at the time of the medium change into the differentiation-inducing medium.

2.3.3 LDH-release assay.

Lactate dehydrogenase (LDH)-release assay was carried out for the quantitative determination of cytotoxicity. 3T3-L1 cells were seeded in 96-well plates at a density of 2 × 104 cells/well in culture medium. After incubation, the cells were treated with BITC for 3 days. LDH activity was measured by using an LDH-Cytotoxicity Test Wako, in accordance with the manufacturer's instructions. The absorbance was measured at 560 nm. Total LDH release (100%) was obtained by the treatment of 0.1% Tween 20.

2.3.4 Oil Red O staining

The matured differentiated 3T3-L1 cells in 12-well plates were washed twice with ice-cold PBS and fixed with 10% formalin for 10 min at room temperature. After removing formalin, the fixed cells were treated with 60% isopropanol for 1 min. The cells were stained with a 60% Oil Red O solution for 15 min at room temperature. After removing the Oil Red O solution, the cells were washed with 60% isopropanol and PBS. The oil droplets present in the stained cells were then dissolved in isopropanol, and absorbance of the dissolved solution was measured at 490 nm using a microplate spectrophotometer.

2.3.5 RT-PCR analysis

The post-confluent 3T3-L1 cells were treated with BITC in the differentiation-inducing medium for 3 days. The total RNA was then extracted using Trizol reagent according to the manufacture's manual. The total RNA was reverse transcribed to cDNA using ReverTra Ace. PCR amplification was then performed with Taq polymerase. Primers used in PCR amplification are as follows: PPAR-γ, 5'-TATggAgTTCATgCTTgTgA-3' and 5'-CgggAAggACTTTATgTATg-3' (316bp); C/EBPα, 5'-gAAggAACTTgAAgCACAAT-3' and 5'-gACACAgAgACCAgATACAA-3' (111bp); C/EBPβ, 5'-CAAgCTgAgCgACgAgTACA-3' and 5'-CAgCTgCTCCACCTTCTTCT-3' (137bp); C/EBPδ, 5'-gATCTgCACggCCTgTTgTA-3' and 5'-CTCCACTgCCCACCTgTCA-3' (148bp); GLUT4, 5'-TCCTTCTATTTgCCgTCCTC-3' and 5'-TgTTTTgCCCCTCAgTCATT-3' (bp); and β-Actin 5'-CTCACCCACACTgTgCCCATCTA-3' and 5'-gCAATgCCAgggTACATggTggT-3' (454bp). The cycles and annealing temperatures used in PCR amplification are as follows: PPAR-γ, 22 cycles, 60ºC; C/EBPα, 25 cycles, 59ºC; C/EBPβ, 30 cycles, 64ºC; C/EBPδ, 25 cycles, 67ºC; GLUT4, 30 cycles, 64ºC; and β-Actin 16 cycles, 65ºC. The PCR products were separated on an agarose gel (2-3%), stained with ethidium bromide, and visualized under UV light. The relative densities of the bands were measured using an Image J Software Program (National Institutes of Health, Bethesda, MD, USA).

2.3.6 Western blot analysis

The post-confluent 3T3-L1 cells were treated with BITC in the differentiation-inducing medium for 2 or 3 days. The whole cell lysates were then prepared in lysis buffer (20 mM Tri-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM NaH_2PO_4, 10 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1% sodium dodecyl sulfate, 1% sodium deoxycholate and 1% Triton X-100) containing

protease inhibitor cocktail and left on ice for 20 min. After sonication, the lysates were centrifuged, and the supernatant was used as the whole cell lysates. The protein concentration in the supernatant was determined by the Bio-Rad protein assay. Equal quantities of protein were subjected to SDS-PAGE and transferred to Immobilon-P membranes. The membranes were blocked, then incubated with the primary antibody overnight at 4ºC followed by an appropriate secondary antibody. The secondary antibody binding was visualized using a Chemi-Lumi One Super (Nacalai Tesque). Densitometric analysis of the bands was carried out using the Image J Software Program.

2.3.7 Glucose uptake assay

The glucose uptake assay was performed using an enzyme-dependent fluorometric assay as previously reported (Yamamoto N et al., 2015). After the treatment with BITC or Comp. C in the differentiation-inducing medium for 3 days, the cells were washed twice with Krebs-Ringer-Hepes-bicarbonate (KRH) buffer containing 0.1% BSA. The cells were incubated with KRH buffer containing 0.1% BSA and 2 mM 2-deoxy-D-glucose (2-DG) for 20 min in a 5% CO_2 incubator. The cells were then washed twice with KRH buffer containing 0.1% BSA, added 0.1 M NaOH to each well and mixed using a plate mixer. The plate was heated to 75ºC for 1 h using a laboratory heating oven. After heating, the lysate in the wells were neutralized by adding 0.1 M HCl. followed by the addition of 50 μL of 200 mM TEA buffer (pH 8.1). The cell lysate (10 μL) was transferred to a 96‐well plate for fluorescence measurement and incubated for 45 min with 100 μL of assay cocktail, containing 50 mM KCl, 0.1 mM $NADP^+$, 20 U/mL of glucose-6-phosphate dehydrogenase, 0.2 units/mL of diaphorase, 5 μM resazurin sodium salt and 50 mM TEA buffer (pH 8.1). The resulting fluorescence was measured using a fluorescence microplate reader (λex 530 nm, λem 590 nm). Quantification of 2-DG-6-phosphate was carried out by

comparing the fluorescence intensity from the experimental samples to its standard curve.

2.3.8 Statistical analysis

All values were expressed as means ± SD. Statistical significance was analyzed by Student's t-test or one-way ANOVA followed by Tukey's HSD using XLSTAT software.

2.4 Results

2.4.1 Effect of BITC on lipid accumulation in preadipocytes

The tested concentration of BITC was determined to be at most 5 μM, because the cytotoxic effect of BITC toward 3T3-L1 was observed at more than 10 μM in an LDH-release assay (Fig. 2.1). To investigate the modulating effect of the BITC treatment on the lipid accumulation in 3T3-L1 preadipocytes, the cells were incubated in the differentiation-inducing medium for 3 days (early stage), then in the differentiation-maintaining medium for additional 3 days (late stage). The cells were treated with or without BITC for 6 days (both stages). Thereafter, the cells were cultured in DMEM containing 10% FBS for 2 days for maturing adipocytes. The intracellular lipid accumulation was measured by Oil Red O staining. As shown in Figs. 2.2 A and 2.2 B, lipid accumulation was significantly inhibited by the BITC treatment all through both stages. We next examined whether the treatment of BITC during the early stage or late stage modulates the lipid accumulation. As shown in Figs. 2.2 C and 2.2 D, the treatment of BITC during the early stage significantly inhibited the lipid accumulation, whereas that during the later stage showed a tendency to enhance the lipid accumulation. These results suggested that its treatment during the early stage plays an important role in modulation of the lipid accumulation in the differentiating preadipocytes. In addition, another ITC compound, AITC, showed a weaker effect on

the lipid accumulation in the differentiation-inducing (early) stage (Fig. 2.2 E) compared to that of BITC (Fig. 2.2 C).

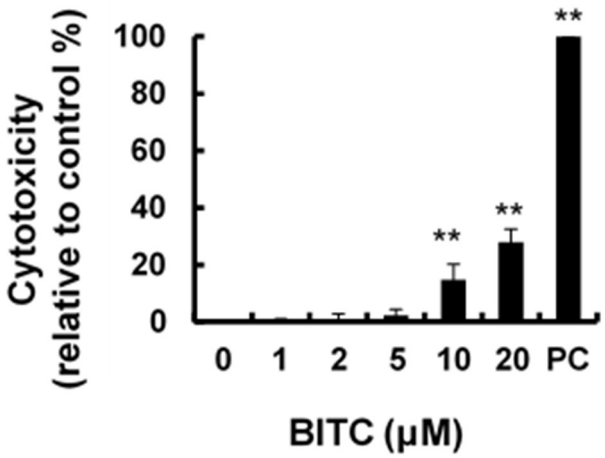
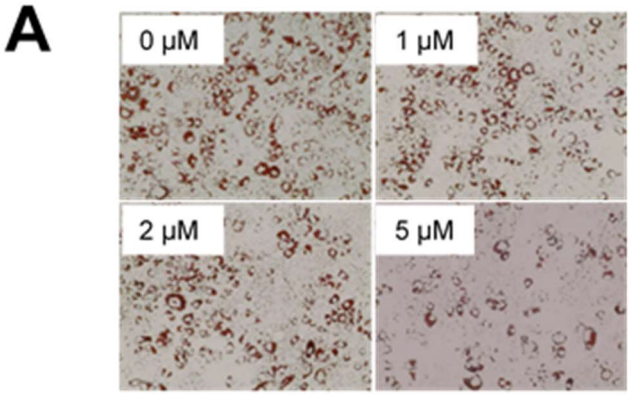
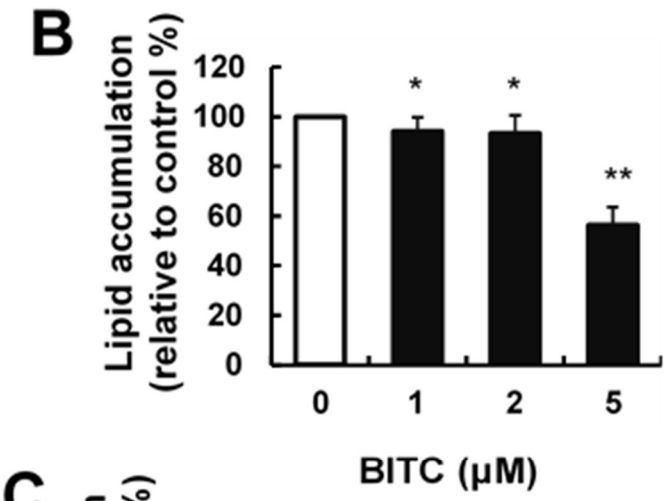
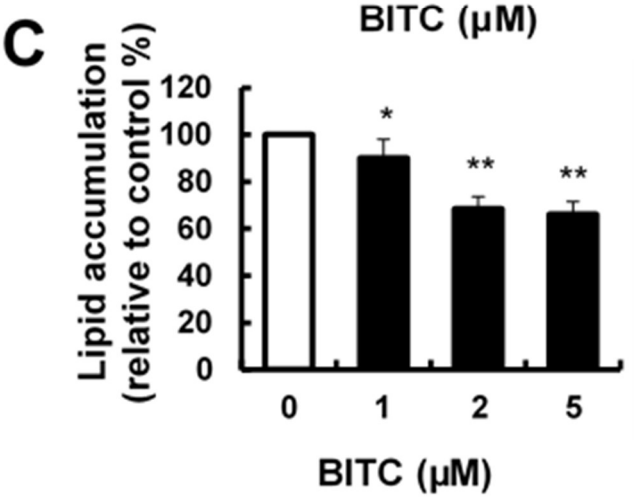
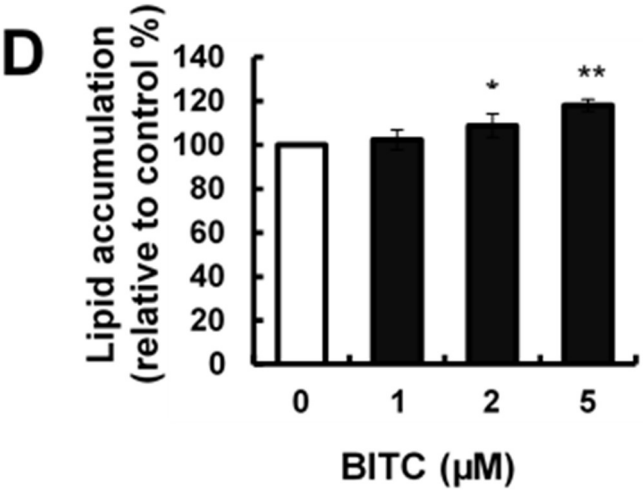


Figure 2.1 Cytotoxic effect of BITC in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were incubated in induction medium with 0.2% Tween-20 as the positive control (PC) or indicated concentrations of BITC for 3 days. The cytotoxicity was determined by an LDH-release assay. All values were expressed as means ± SD of three separate experiments (*$p < 0.05$, **$p < 0.01$ compared to negative control).




B
Lipid accumulation
(relative to control %)
120
100
80
60
40
20
0
*
*
**
0
1
2
5
BITC (μM)


C
Lipid accumulation
(relative to control %)
120
100
80
60
40
20
0
*
**
**
0
1
2
5
BITC (μM)


D
Lipid accumulation
(relative to control %)
140
120
100
80
60
40
20
0
*
**
0
1
2
5
BITC (μM)

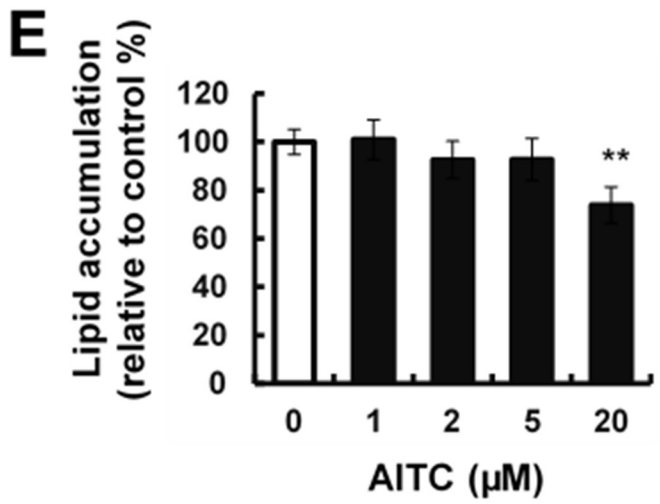
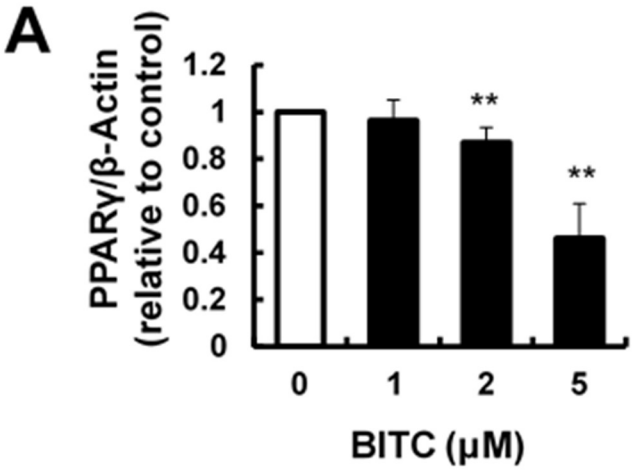


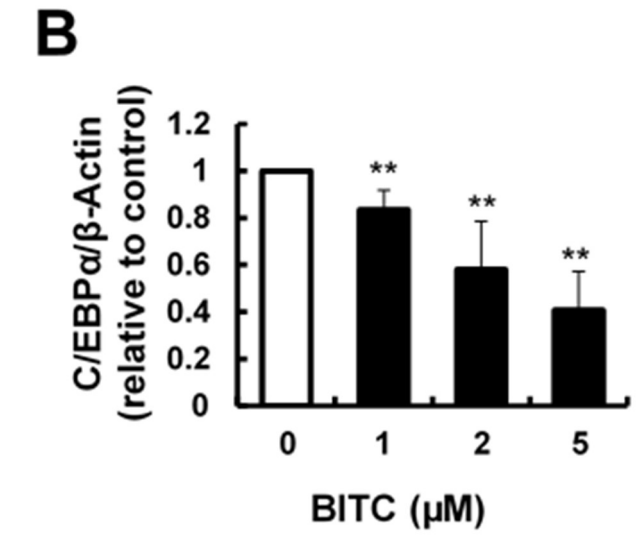
Figure 2.2 Modulating effects of BITC on lipid accumulation in 3T3-L1 adipocytes. The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media for 3 days, then incubated in the differentiation-maintaining media for another 3 days. BITC was treated with the cells all through both stages for 6 days. Thereafter, the cells were cultured in the completed media for 2 days (A, B). (C) The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media with or without BITC for 3 days (early stage treatment), then incubated in the differentiation-maintaining media for 3 days and in the completed media for 2 days. (D) The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media for 3 days, then incubated in the differentiation-maintaining media with or without BITC (late stage treatment) for 3 days and in the completed media for 2 days. (E) The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media with or without AITC for 3 days (early stage treatment), then incubated in the differentiation-maintaining media for 3 days and in the completed media for 2 days. The lipid accumulation level was determined by Oil Red O staining after an 8-day incubation.

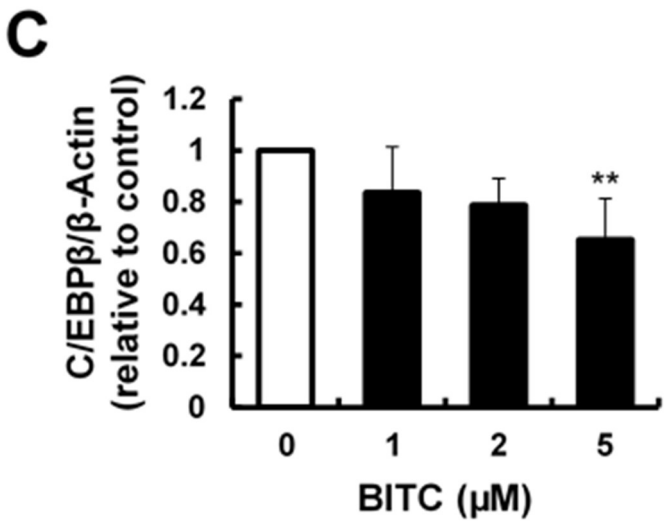
All values were expressed as means ± SD of three separate experiments (*p < 0.05, **p < 0.01 compared to negative control).

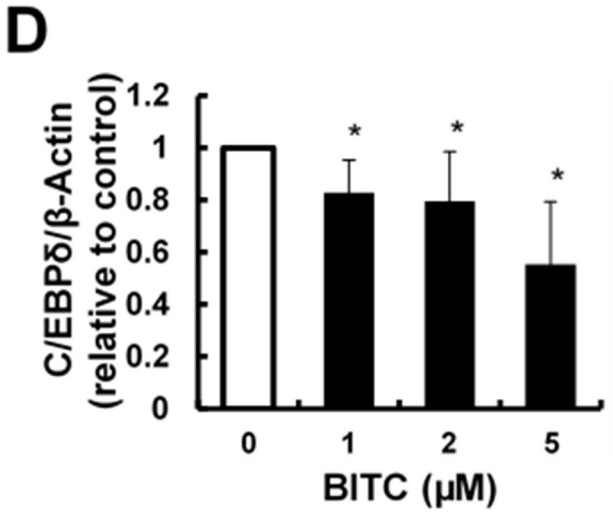
2.4.2 Effect of BITC on adipogenic markers gene expression

To determine whether BITC modulates the master markers of the adipocyte-specific differentiation, the expression levels of PPAR-γ and C/EBPα were determined by RT-PCR using the 3T3-L1 cells incubated in the differentiation-inducing medium with or without BITC for 3 days. The expression level of PPAR-γ was significantly decreased by 2 and 5 μM BITC to 87.0% and 46.1% of the control level, respectively. The C/EBPα expression level was significantly decreased at the concentrations from 1 μM BITC and higher. We next checked the effect of BITC on the early differentiation markers such as C/EBPβ and C/EBPδ. The expression level of C/EBPβ was significantly decreased by 5 μM BITC to 65.3% of the control level. The C/EBPδ level was significantly decreased at the concentrations from 1 μM BITC and higher. These results suggested that BITC could negatively regulate the gene expression of the adipogenic transcriptional factors in 3T3-L1 preadipocytes during the differentiation-inducing stage.




B
C/EBPα/β-Actin
(relative to control)
1.2
1
0.8
0.6
0.4
0.2
0
**
**
**
0
1
2
5
BITC (μM)


C
C/EBPβ/β-Actin
(relative to control)
1.2
1
0.8
0.6
0.4
0.2
0
**
0
1
2
5
BITC (μM)


D
C/EBPδ/β-Actin
(relative to control)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0
1
2
5
BITC (μM)

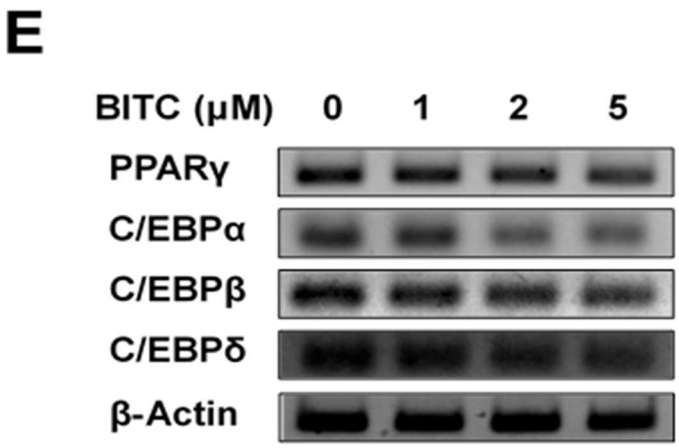
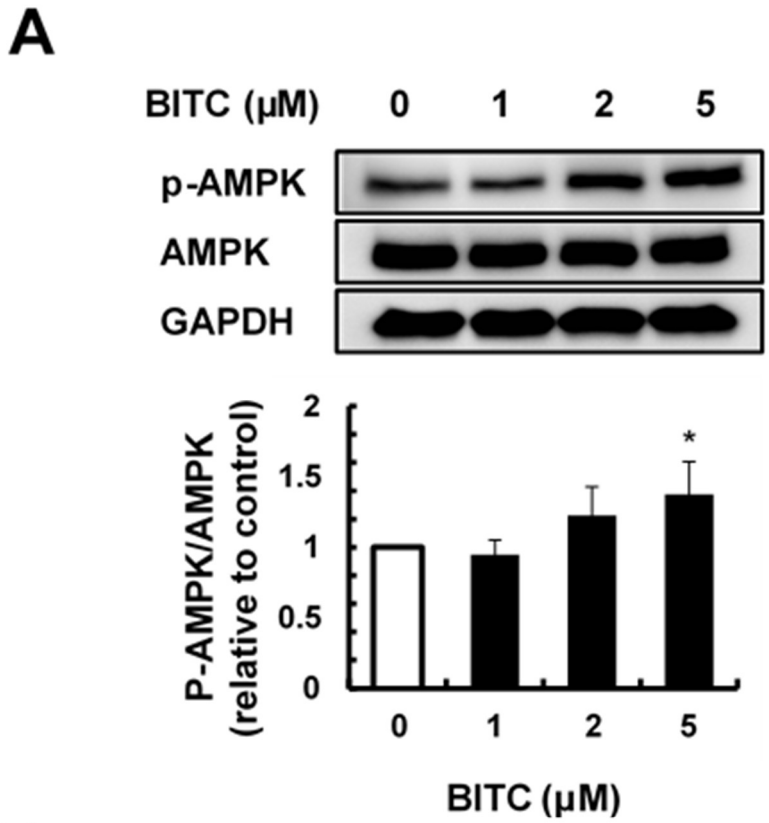


Figure 2.3 Suppression of the gene expression of adipogenic transcriptional factors by BITC. The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media with or without BITC for 3 days (early stage treatment), then the gene expression level was determined by RT-PCR. Quantitative data for (A) PPARγ, (B) C/EBPα, (C) C/EBPβ, and (D) C/EBPδ, and representative blots (E). All values were expressed as means ± SD of three separate experiments (*$p < 0.05$, **$p < 0.01$ compared to negative control).

2.4.3 Effect of BITC on AMPK and ERK phosphorylation

Since AMPK is known to be involved in the negative regulation of adipogenesis (Carling D et al., 2011), we examined whether the AMPK activation was involved in the modulating effect of BITC on the lipid accumulation. As shown in Fig. 2.4A, the treatment of 5 μM BITC during the early stage for 3 days significantly increased the phosphorylation level of AMPK to 138% of the control level. In addition, the effect of BITC on the phosphorylation level of ERK1/2, one of the other protein kinases to regulate the adipocytes differentiation (Sale EM et al., 1995), was determined by Western blotting. As shown in Fig. 2.4B, the phosphorylation level of ERK1/2 was

significantly decreased by even 1 μM BITC for 2 days after treatment.



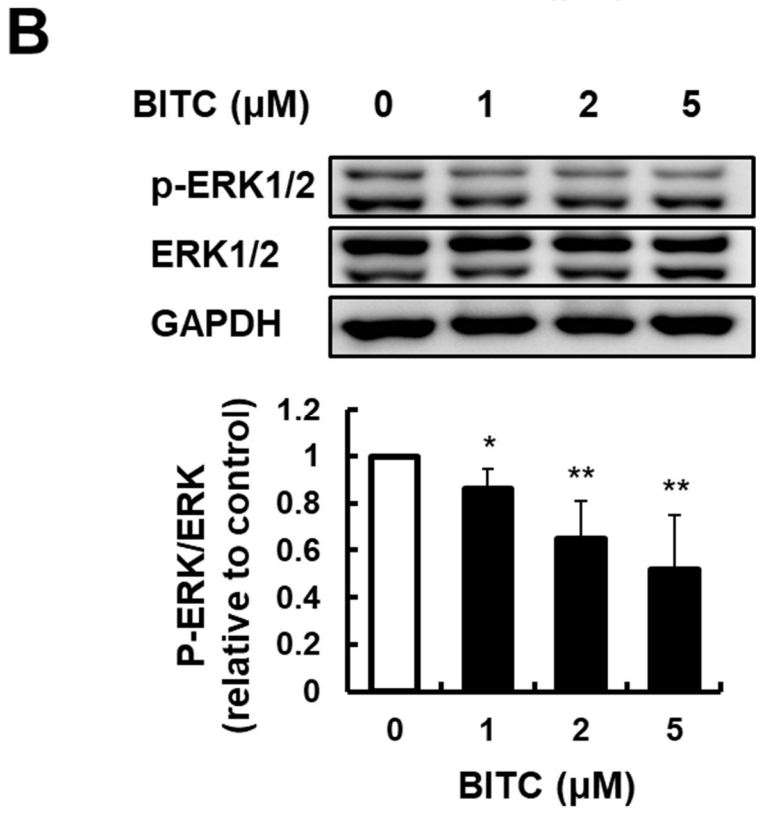


Figure 2.4 Modulating effect of BITC on the phosphorylation of AMPK and ERK1/2. The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media with or without BITC for 3 days for the phosphorylated AMPK (A) or for 2 days for the phosphorylated ERK1/2 (B), then the protein expression level was determined by Western blotting. All values were expressed as means ± SD of three separate experiments (*$p < 0.05$, **$p < 0.01$ compared to negative control).

2.4.4 Effect of BITC on glucose uptake

The expression level of GLUT4 was next determined, because it is one of the main target genes of C/EBPα and plays a critical role in glucose uptake by adipocytes (Kaestner KH et al., 1990). As shown in Fig. 2.5A, the expression level of GLUT4 was significantly decreased by the treatment of 2 or 5 μM BITC (70.4% or 62.7% of the control level, respectively) during the early stage for 3 days. Thus, the effect of BITC on the glucose uptake was next examined. The glucose uptake in the BITC-treated 3T3-L1 cells was evaluated by an enzyme-dependent fluoremetoric assay with 2-DG, a widely-used and non-metabolizable glucose tracer (Yamamoto N et al., 2015). As shown in Fig. 2.5B, the treatment of 5 μM BITC during the early stage for 3 days significantly suppressed the intracellular 2-DG level. These results suggested that BITC might suppress the glucose uptake by the GLUT4 down-regulation.

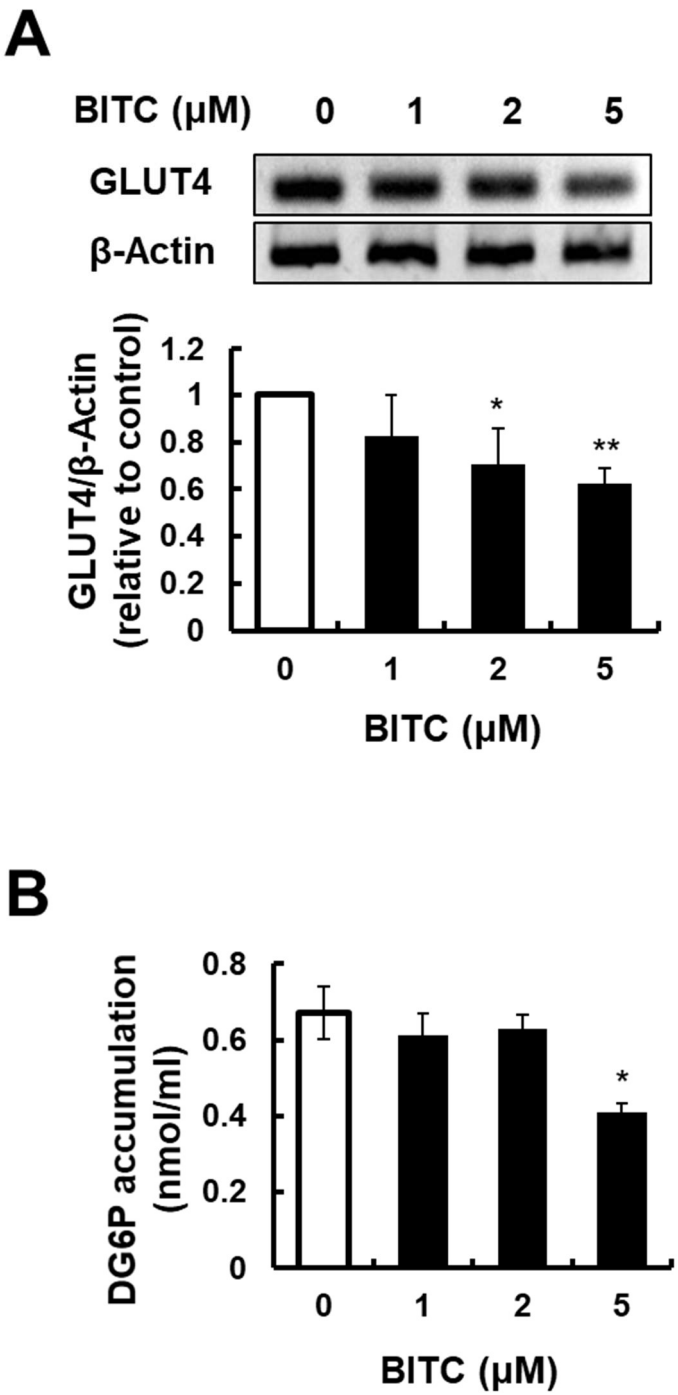


Figure 2.5 Inhibitory effects of BITC on the GLUT4 gene expression and glucose uptake. The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media with or without BITC for 3 days, then the gene expression level of

GLUT4 was determined by RT-PCR (A) or the glucose uptake level was determined by an enzyme-dependent fluorometric assay (B). All values were expressed as means ± SD of three separate experiments (*$p < 0.05$, **$p < 0.01$ compared to negative control).

2.4.5 Effect of Comp. C on glucose uptake and lipid accumulation

To confirm the involvement of AMPK in BITC-inhibiting adipocyte differentiation accurately, we employed Compound C (AMPK inhibitor, 2 μM) to the preadipocytes. We have found that 5 μM BITC decrease the GLUT4 gene expression, glucose uptake and lipid accumulation in adipocyte. Thus, we determined the effect of Comp. C on BITC-induced inhibition of glucose uptake and lipid accumulation. We found that Comp. C significantly altered BITC-induced inhibition of glucose uptake in preadipocytes (Fig.2.6A). In addition, Comp. C also significantly altered BITC-induced inhibition of lipid accumulation (Fig.2.6B). Compound C alone significantly enhanced the basal level of 2-DG in the 3T3-L1 cells, whereas it did not affect the basal level of lipid accumulation. These results suggested that AMPK might, at least partly, be involved in the mechanism underlying the suppression of the glucose uptake and lipid accumulation by BITC.

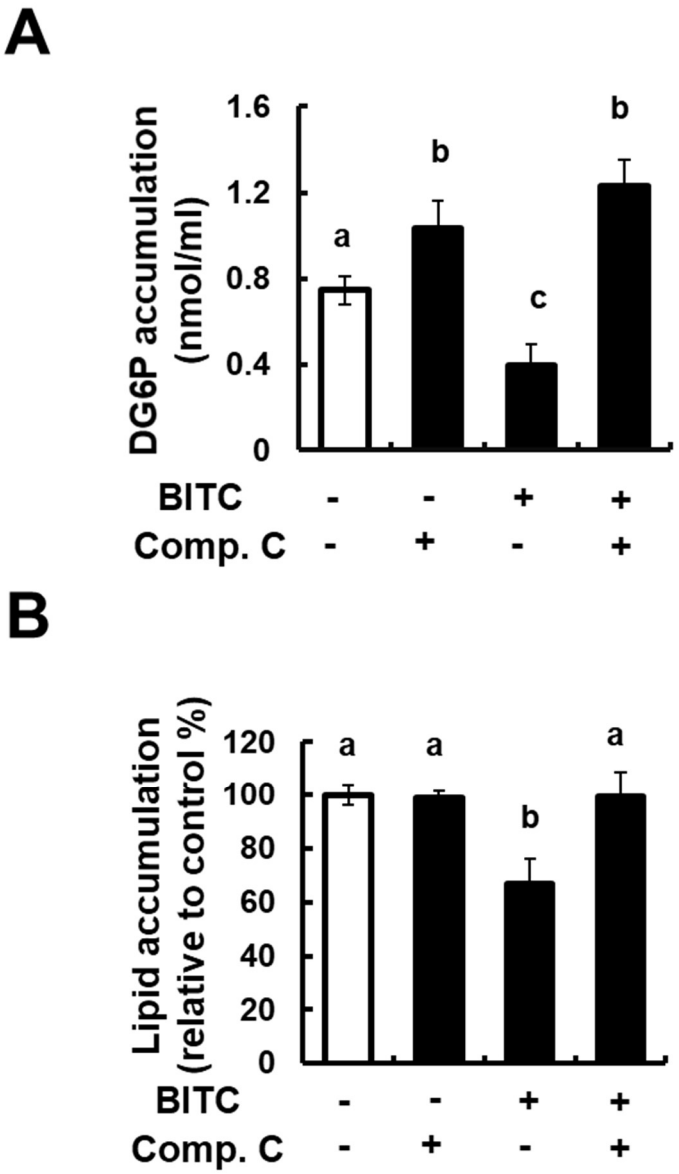


Figure 2.6 AMPK inhibitor enhanced the BITC-induced inhibitory of glucose uptake and lipid accumulation. The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media treated with 5 μM BITC in the presence or absence of 2 μM for 3 days, then the glucose uptake level was determined by an enzyme-dependent fluorometric assay (A) or the cells were stained with Oil Red O at 8 days (B). The values represent means ± S.D. of three separate experiments. Data were analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among treatments for

each compound ($p < 0.05$).

2.5 Discussion

In this study, we demonstrated that BITC is a potential inhibitor of the lipid accumulation in the differentiating preadipocytes. BITC improves the high-fat diet-induced liver fat accumulation in vivo (Alsanea &Liu, 2017), thus supporting our findings. Although the continuous treatment of BITC during all the stages (differentiation-inducing, different-maintaining and maturing stages) has also been reported to inhibit the lipid accumulation (Kim et al., 2015), its molecular mechanism remains to be clarified. The BITC treatment during the early stage, but not in the late stage, contributed to suppression of the lipid accumulation in the differentiating 3T3-L1 preadipocytes (Fig. 2.2C). AITC, an aliphatic ITC, has the potential for inhibition of the adipocyte differentiation, which requires the relatively higher concentrations (15-30 μM) and longer incubation (8 days) (Lo et al., 2018). Our findings (Figs. 2.2C and 2.2E) supported the lower potency of AITC than that of BITC. Sulforaphane, a different aliphatic ITC, is also effective for lipid accumulation at concentrations between 5 and 20 μM mainly through inhibition of the cell mitotic expansion (Choi et al., 2012). Esculetin, a coumarin compound derived from medicinal plants, inhibits adipogenesis by down-regulating the PPARγ signaling in 3T3-L1 cells at a concentration of more than 50 μM (Shin et al., 2010). A soybean isoflavone, genistein, shows an inhibitory effect on the lipid accumulation at 50 μM (Zhang et al., 2009). Therefore, BITC is one of the most potent ITCs for inhibiting the lipid accumulation in 3T3-L1 preadipocytes, equivalent to the Ashitaba chalcones (Zhang et al .2013).

BITC inhibited the gene expressions of the adipogenic transcriptional factors and the phosphorylation level of ERK1/2 with the increased level of AMPK phosphorylation (Figs. 2.3 and 2.4). C/EBPα and PPARγ are the master regulators for adipogenesis, controlling the adipocyte differentiation through the expression of a

variety of other transcriptional factors, such as fatty acid synthase, adipocyte fatty acid binding protein, GLUT4, and adiponectin, whose function leads to the formation of mature adipocytes (Wu et al. 1999). PPARγ is heterodimerized with retinoic acid X-receptor (Kliewer et al., 1992) to regulate the transcription of the adipocyte-specific genes (Wu et al. 1999). C/EBP-α is most abundant in mature adipocytes and mainly contributes to the enhanced glucose uptake (Wu et al. 1999). C/EBPβ and C/EBPδ are transiently expressed during early differentiation into adipocytes to increase the expression of other adipogenic transcriptional factors such as C/EBPα and PPAR-γ (Yeh et al., 1995). Thus, BITC might suppress the C/EBPβ and C/EBPδ expressions at the earlier stage and subsequently suppress C/EBPα and PPAR-γ during the adipocyte differentiation. The expression of GLUT4, playing a critical role in glucose uptake in adipocytes, is regulated by C/EBPα during the adipocyte differentiation (Wu et al. 1999). BITC suppressed the gene expression of GLUT4 (Fig. 2.5A), and also actually inhibited the glucose uptake (Fig. 2.5B). These results suggested that inhibition of the adipocyte differentiation and glucose uptake might play an important role in the inhibition of the lipid accumulation by BITC in 3T3-L1 preadipocytes.

Comp. C (an AMPK inhibitor) significantly altered BITC-induced inhibition of glucose uptake and lipid accumulation (Fig. 2.6). AMPK plays critical roles in modulating many biological pathways including the lipid and glucose metabolism (Carling et al., 2011). ACC, a molecular target of AMPK (Carling et al., 2011), is an essential enzyme for the synthesis of fatty acids. In addition, an AMPK inhibitor cancels the down-regulation of C/EBPα, C/EBPβ and PPARγ induced by the Ashitaba chalcones (Zhang et al., 2013). Thus, inhibition of the adipocytes differentiation by BITC might involve the enhanced phosphorylation of AMPK. This idea was strongly supported by the finding that Compound C, the AMPK inhibitor, abolished the inhibitory effects of BITC on glucose uptake and lipid accumulation in 3T3-L1 preadipocytes (Fig. 2.6). These results suggested that the AMPK activation might, at

least partly, contribute to the suppression of the glucose uptake and lipid accumulation by BITC, possibly through down-regulation of C/EBPs and PPARγ. In addition, attenuation of the ERK1/2 activation in preadipocytes led to a decrease in the adipocytes differentiation (Sale et al., 1995). However, there is a controversial report showing that the activation of ERK1/2 negatively regulated the adipocyte differentiation by the phosphorylation of PPARγ (Hu et al., 1996). The present results implied that the inhibition of the ERK1/2 phosphorylation by BITC during the early stage might contribute to the inhibitory effects on the adipocyte differentiation.

In conclusion, the present study provides evidence that BITC is one of the potential natural agents for the prevention against not only obesity, but also obesity-related chronic diseases, because adipocyte differentiation is associated with the pathogenesis and progression of type 2 diabetes, hypertension, and cardiovascular disease (Ntambi & Young-Cheul., 2000). Further studies are necessary to elucidate its more precise mechanisms of anti-adipogenesis not only using in vitro models, but also using in vivo animal models in terms of involvement of the adipocyte differentiation.

CHAPTER 3

Benzyl isothiocyanate promotes glucose uptake via activation PI3K/Akt signaling pathway in the tumor necrosis factor-alpha-induced insulin resistance 3T3-L1 adipocytes

3.1 Abstract

Insulin resistance is characterized by reduce glucose uptake by target tissue or cell owing to impaired insulin signaling transduction and/or glucose transporting ability. Besides, enlarged adipocytes secret proinflammatory factors, such as tumor necrosis factor-alpha (TNF-α), which lead to insulin resistance. The treatment of BITC significantly improved glucose uptake in mature adipocytes. BITC activated both PI3K/Akt signaling pathway and MAPKs, including ERK1/2 and JNK1/2 but not p38 in mature adipocytes. Inhibitory experiments using an inhibitor, LY294002, significantly attenuated the BITC-stimulated glucose uptake, lipid accumulation and the activation of PI3K/Akt pathway in mature adipocytes. However, PD98059, an MEK inhibitor, inhibited BITC-stimulated ERK phosphorylation but neither deletes BITC-stimulated glucose uptake nor lipid accumulation in mature adipocytes. Furthermore, BITC also significantly increased glucose uptake in the TNF-α-induced insulin resistance adipocytes. These results suggest that the BITC induced glucose uptake and lipid accumulation in mature 3T3-L1 adipocytes are involved in activation PI3K/Akt signaling pathway.

3.2 Introduction

Insulin resistance, which is refers to a reduction in target cell metabolic response to insulin or an impaired effect of injected or circulating insulin on blood glucose at the whole body, is highly correlated with obesity and diabetes in experimental animals and humans (Kahn et al., 1991). Insulin increases glucose uptake muscle and fat, and

inhibits hepatic glucose production, thus serving as the primary regulator of blood glucose concentration (Saltiel et al., 2001). Insulin resistance leads to dysregulation of this process, thereby causing a high level of glucose in blood. Insulin resistance has also been described in various diseases, such as certain cancers, cardiovascular disease, atherosclerosis and hypertension (Stolzenberg et al., 2005; Rose et al., 2004).

Tumor necrosis factor alpha (TNF-α) is an inflammatory cytokine, which is secreted chiefly by activated macrophages. Numerous recent studies have implicated the involvement of TNF-α in insulin resistance in mice and human (Vinolo et al., 2012; Sakurai et al., 2012). In addition, administration of TNF-α has been proved to stimulate an insulin resistance in adipocytes (Stephens et al., 1997). It has been suggested that TNF-α induces insulin resistance in terms of glucose uptake in adipocytes because of the activation of mitogen-activated protein kinases (MAPKs) which inhibit insulin receptor signaling at the tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1), thereby impairing the activation of phosphatidyl inositol-3-phosphate kinase (PI3K) which play a major role in insulin function (Gual et al., 2005; Tanti & Jager, 2009). Down-regulation of insulin mediated PI3K/protein kinase B (Akt) signaling transduction has been proven to be inhibited glucose transporter 4 (GLUT4) translocation and reduced glucose uptake in adipocytes (Kong et al., 2013).

3.3 Materials and Methods

3.3.1 Materials

See Chapter 2

3.3.2 Cell culture and treatments

See Chapter 2

3.3.3 Establishment of insulin resistance adipocyte model

The cells had the morphological and biochemical properties of adipocytes were chronically exposed to 10 ng/mL TNF-α which was dissolved in DDW containing 0.1% fatty acid-free and growth factor-depleted bovine serum albumin for 4 days. During the treatment period, fresh TNF-α was changed every day.

3.3.4 Western blot analysis

The post-confluent 3T3-L1 cells were treated with BITC or inhibitors in the differentiation-maintaining medium. The whole cell lysates were then prepared in lysis buffer (20 mM Tri-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM NaH_2PO_4, 10 mM NaF, 2 mM Na_3VO_4, 1 mM phenyl methyl sulfonyl fluoride, 1% sodium dodecyl sulfate, 1% sodium deoxycholate and 1% Triton X-100) containing protease inhibitor cocktail and left on ice for 20 min. After sonication, the lysates were centrifuged, and the supernatant was used as the whole cell lysates. The protein concentration in the supernatant was determined by the Bio-Rad protein assay. Equal quantities of protein were subjected to SDS-PAGE and transferred to Immobilon-P membranes. The membranes were blocked, then incubated with the primary antibody overnight at 4^{o}C followed by an appropriate secondary antibody. The secondary antibody binding was visualized using a Chemi-Lumi One Super (Nacalai Tesque). Densitometric analysis of the bands was carried out using the Image J Software Program.

3.3.5 Glucose uptake assay

The glucose uptake assay was performed using an enzyme-dependent fluorometric assay as previously reported. After the treatment with BITC or inhibitors in the differentiation-maintaining medium, the cells were washed twice with Krebs-Ringer-Hepes-bicarbonate (KRH) buffer containing 0.1% BSA. The cells were

incubated with KRH buffer containing 0.1% BSA and 2 mM 2-deoxy-D-glucose (2-DG) for 20 min in a 5% CO_2 incubator. The cells were then washed twice with KRH buffer containing 0.1% BSA, added 0.1 M NaOH to each well and mixed using a plate mixer. The plate was heated to 75ºC for 1 h using a laboratory heating oven. After heating, the lysate in the wells were neutralized by adding 0.1 M HCl. followed by the addition of 50 µL of 200 mM TEA buffer (pH 8.1). The cell lysate (10 µL) was transferred to a 96-well plate for fluorescence measurement and incubated for 45 min with 100 µL of assay cocktail, containing 50 mM KCl, 0.1 mM $NADP^+$, 20 U/mL of glucose-6-phosphate dehydrogenase, 0.2 units/mL of diaphorase, 5 µM resazurin sodium salt and 50 mM TEA buffer (pH 8.1). The resulting fluorescence was measured using a fluorescence microplate reader (λex 530 nm, λem 590 nm). Quantification of 2-DG-6-phosphate was carried out by comparing the fluorescence intensity from the experimental samples to its standard curve.

3.3.6 Statistical analysis

All values were expressed as means ± SD. Statistical significance was analyzed by Student's t-test or one-way ANOVA followed by Tukey's HSD using XLSTAT software.

3.4 Results

3.4.1 Effect of BITC on glucose uptake insulin resistance adipocytes

Treatment of 3T3-L1 adipocytes with insulin increased glucose uptake by 66% (Fig.3.1). However, TNF-α treatment suppressed glucose uptake by 33%. TNF-α treatment suppressed insulin-stimulated glucose uptake by 59%. When cells pretreated with BITC, the glucose uptake impaired by TNF-α was restored.

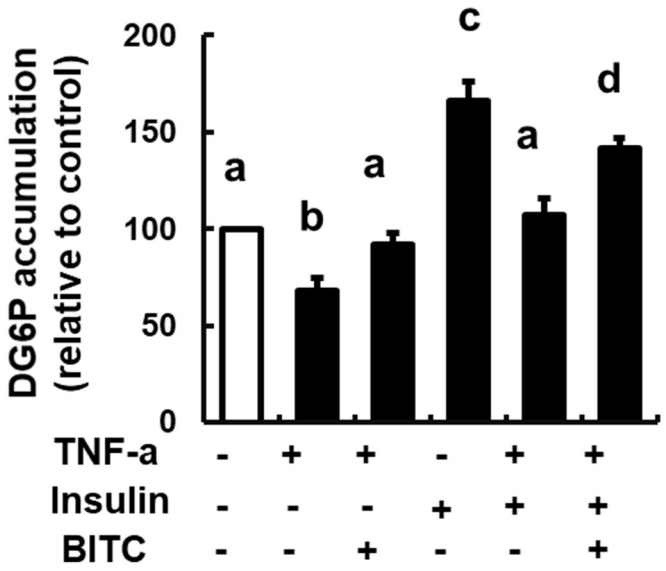


Figure 3.1 BITC enhanced glucose uptake in TNF-α-induced insulin resistance adipocyte. The mature 3T3-L1 adipocytes were incubated with DMEM supplement with 10% FBS with or without 10 μg/ml TNF-α for 4 days and changed medium every day, after 4 h starvation, 5 μM BITC pretreated 1 h, insulin treated 10 min, then the glucose uptake level was determined by an enzyme-dependent fluorometric assay. Data were analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among treatments for each compound ($p < 0.05$).

3.4.2 Effect of BITC on glucose uptake in mature adipocytes.

As BITC enhanced glucose uptake in the insulin resistance adipocytes, we further examined the BITC weather alter glucose uptake in mature adipocytes. To study the effect of BITC on glucose uptake in 3T3-L1 adipocytes, the cells were incubated with 5 μM BITC for 30 min and 2-deoxy-glucose uptake was assayed. In the mature 3T3-L1 adipocytes, 5 μM BITC enhanced intercellular 2-deoxy-D-glucose-phosphate accumulation both with insulin-stimulated and basal (Fig. 3.2). It is indicated that BITC increased both basal and insulin-enhanced glucose uptake in the mature 3T3-L1 adipocytes.

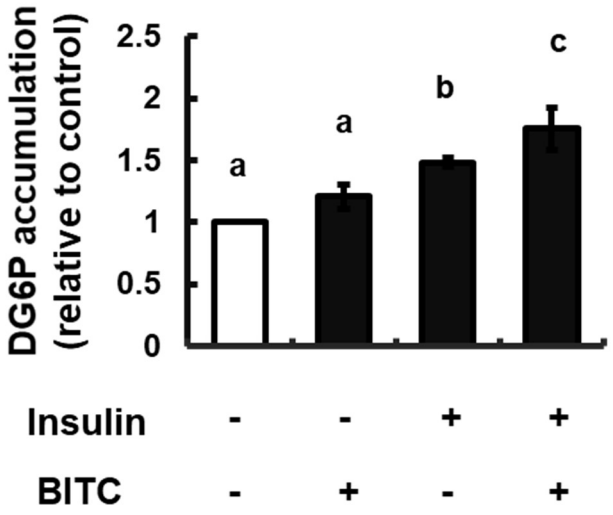
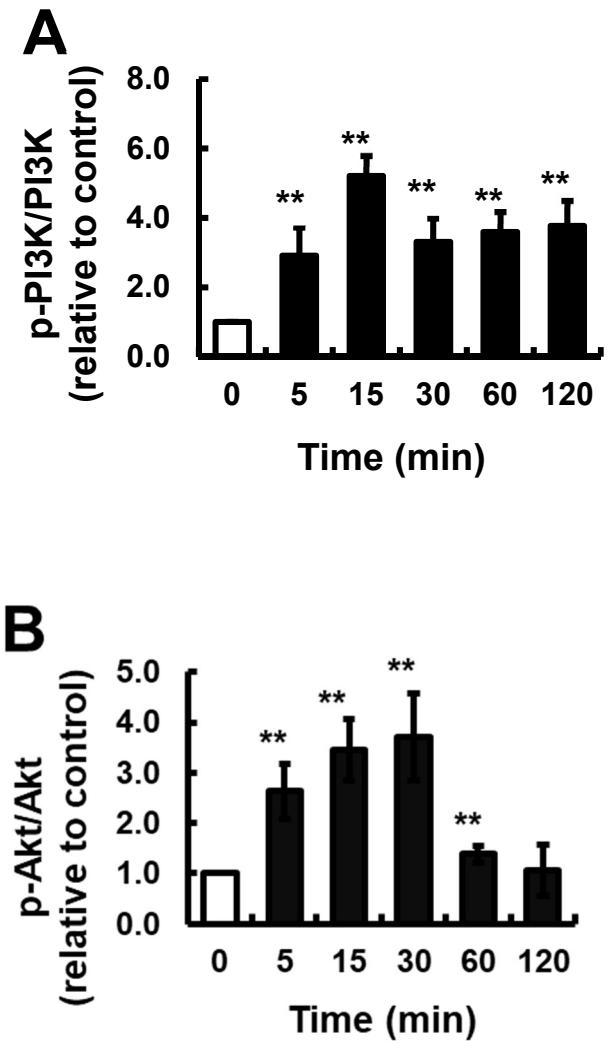


Figure 3.2 BITC increased glucose uptake in mature 3T3-L1 adipocytes. The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media for 3 days, then starvation 5 h. Therefore, BITC or insulin treated 30 min then the glucose uptake level was determined by an enzyme-dependent fluorometric assay. Data were analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among treatments for each compound ($p < 0.05$).

3.4.3 Effect of BITC on PI3K/Akt phosphorylation in mature adipocytes

To investigate the molecular mechanism underlying BITC-stimulated glucose uptake and lipid accumulation, we studied key kinases involved in the insulin signaling pathway. PI3K/Akt signaling pathway plays a major role in insulin-induced glucose and lipid metabolism. To clarify the promotion glucose uptake effect of BITC, we examined the effect of BITC on activation of PI3K and Akt. As the Fig.3.3A showed, BITC increased PI3K phosphorylation by 3-fold relative to control after 5 min. Similarly, BITC significantly increased the activation of Akt by 2.6-fold relative to basal after 5 min treatment (Fig. 3.3B). These results suggest that BITC activates PI3K/Akt pathway in mature 3T3-L1 adipocytes.



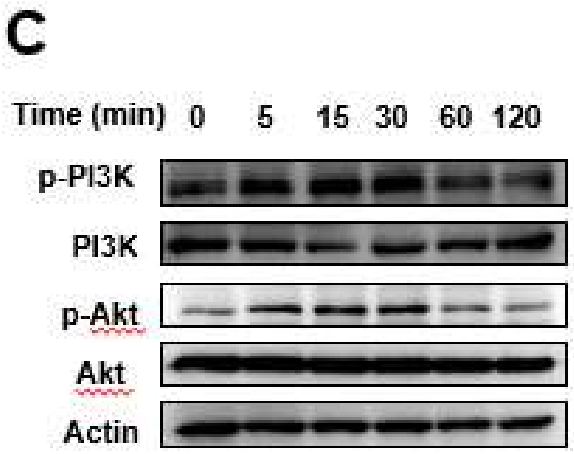
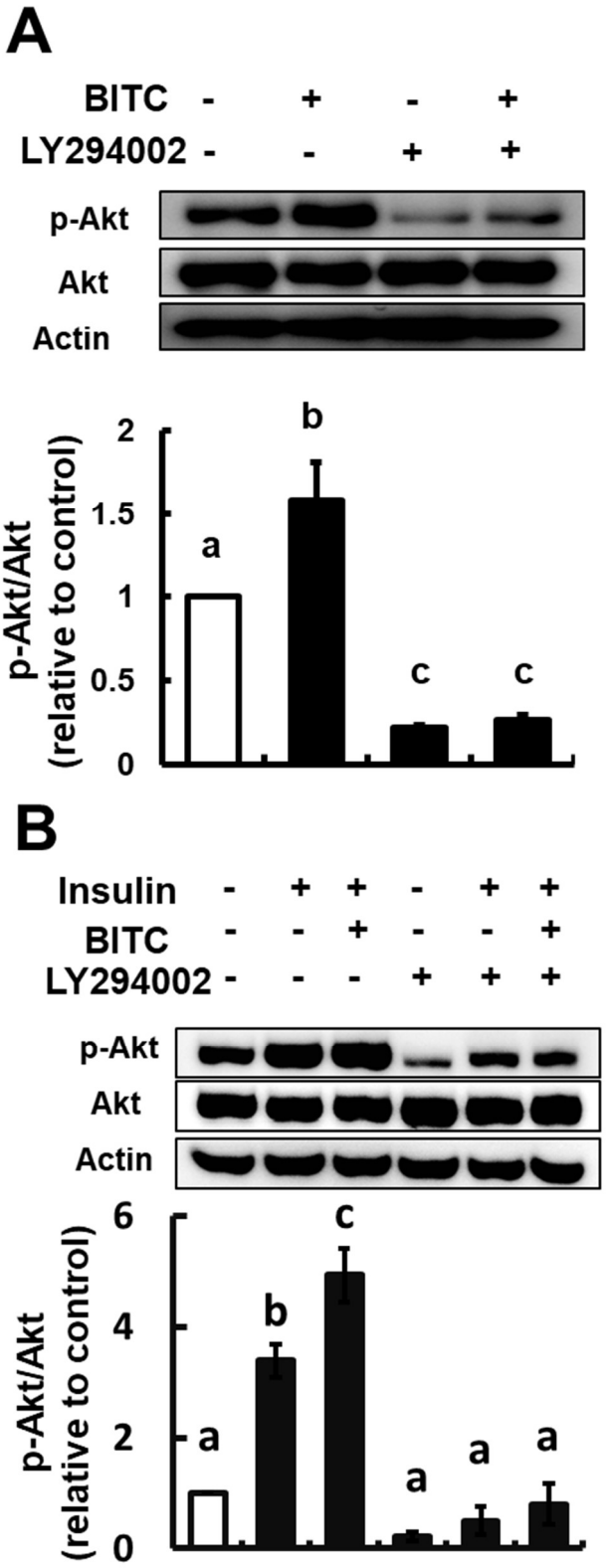


Figure 3.3 BITC activated PI3K/Akt signaling pathway in mature adipocyte. The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media for 3 days, then starvation for 3 h. Thereafter, treatment with 5 μM BITC for indicated time for the phosphorylated PI3K (A) or phosphorylated Akt (B), then the protein expression level was determined by Western blotting. All values were expressed as means ± SD of three separate experiments (*$p < 0.05$, **$p < 0.01$ compared to negative control).

3.4.4 Effect of LY294002 on BITC-stimulated glucose uptake

To investigate the PI3K/Akt pathway was responsible for the BITC-induced improvements in glucose uptake, the cells were pretreated with a PI3K inhibitor LY294002 for 1 h before BITC treatment. BITC increased both basal and insulin-stimulated phosphorylation of Akt at Ser473, and this increase was significantly blocked by LY294002 (Figs.3.4A and 3.4B). In addition, insulin-induced glucose uptake was significantly cancelled by pretreatment of the adipocytes with the PI3K inhibitor LY294002 (Fig.3.4C). As expected, LY294002 also blocked the BITC-stimulated glucose uptake (Fig.3.4C). These results indicated that BITC increases the

glucose uptake through the increment of the PI3K/Akt signaling pathway in mature 3T3-L1 adipocytes.



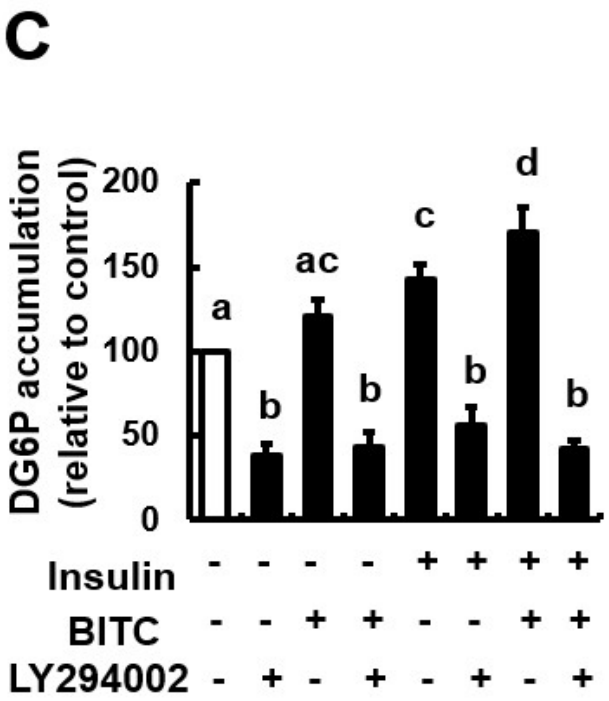
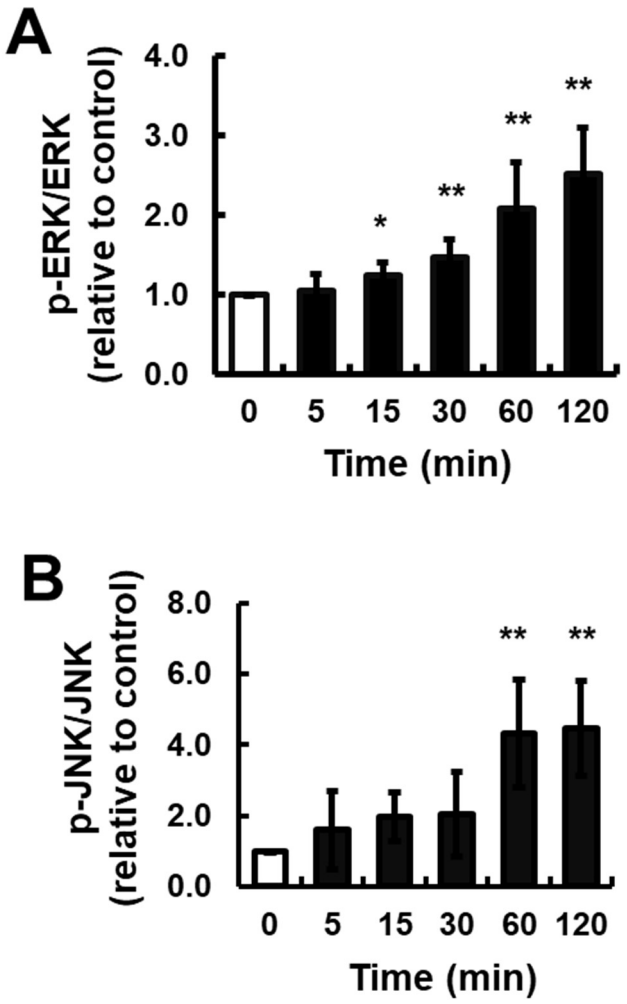


Figure 3.4 LY294002 inhibited both basal and BITC-induced Akt phosphorylation, glucose uptake and lipid accumulation. The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media for 3 days, then starvation for 4 h. Thereafter, 10 μM LY294002 pretreated 1 h and treatment with 5 μM BITC for 30 min for the phosphorylated Akt (A), 10 μM LY294002 pretreated 1 h and treatment with 5 μM BITC for 5 min then insulin treated 10 min for the phosphorylated Akt (B) or glucose uptake, then the protein expression level was determined by Western blotting or the glucose uptake level was determined by an enzyme-dependent fluorometric assay (C). Data were analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among treatments for each compound ($p < 0.05$).

3.4.5 Effect of BITC on MAPK phosphorylation in mature adipocytes

Then we evaluated the contribution of MAPKs to the BITC-induced glucose

uptake and lipid accumulation in mature adipocytes. MAPK/ERK pathway is required for series of metabolic event and is associated with modulation effect on glucose uptake. To investigate the role of the MAPK/ERK pathway in BITC-induced glucose uptake in mature adipocytes, the phosphorylation level of ERK1/2 was estimated. After incubation with 5 μM BITC, western blot results showed that BITC significantly increased ERK1/2 phosphorylation by 2-fold over to control after 60 min in the differentiated 3T3-L1 adipocytes (Fig. 3.5A). Additionally, BITC significantly increased phosphorylation of JNK1/2 by 4-fold compare with control after 60 min (Fig.3.5B). However, p38 was not significantly activated by BITC in mature adipocytes (Fig 3.5C).



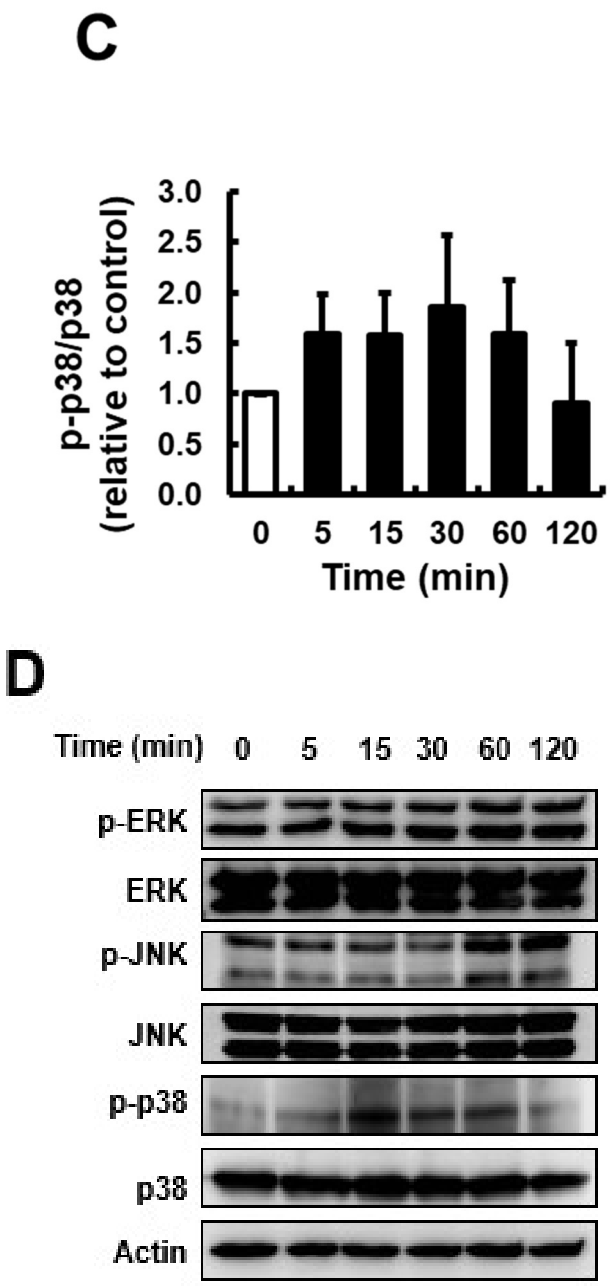
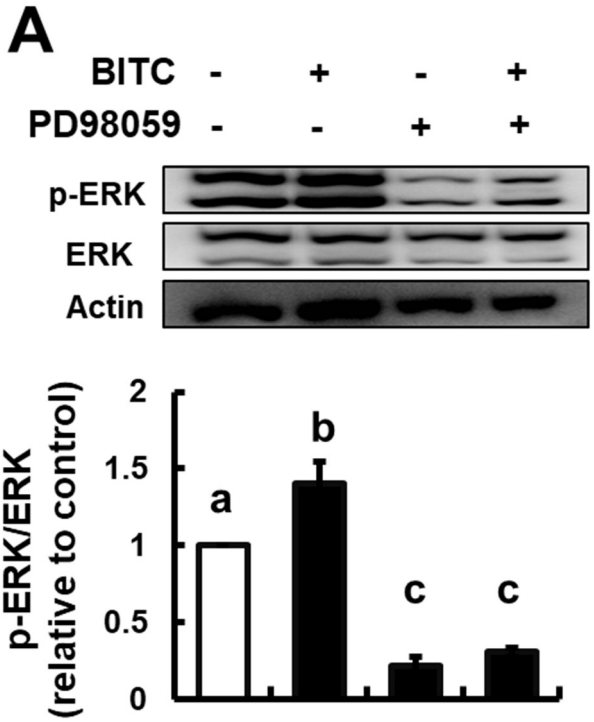


Figure 3.5 BITC activated MAPKs signaling pathway in mature adipocyte. The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media for 3 days, then starvation for 3 h. Thereafter, treatment with 5 μM BITC for indicated time for the phosphorylated ERK (A), phosphorylated JNK (B) or phosphorylated p38 (C), then the protein expression level was determined by Western blotting. All values

were expressed as means ± SD of three separate experiments (*p < 0.05, **p < 0.01 compared to negative control).

3.4.6 Effect of PD98059 on BITC-induced glucose uptake

To confirm the involvement of ERK1/2 in BITC-induced glucose uptake accurately, therefore, we applied an MEK inhibitor PD98059 to block ERK1/2 upstream MEK activity. BITC-induced ERK1/2 phosphorylation was completely cancelled by pretreatment of the adipocytes with the MEK inhibitor PD98059 (Fig.3.6A), however, PD98059 blocked the BITC-induced 2DG6P accumulation intercellular (Fig.3.6B) the mature 3T3-L1 adipocytes. These results indicate that MAPK/ERK signaling was not a major pathway involved in the BITC-induced glucose uptake in the mature 3T3-L1 adipocytes.



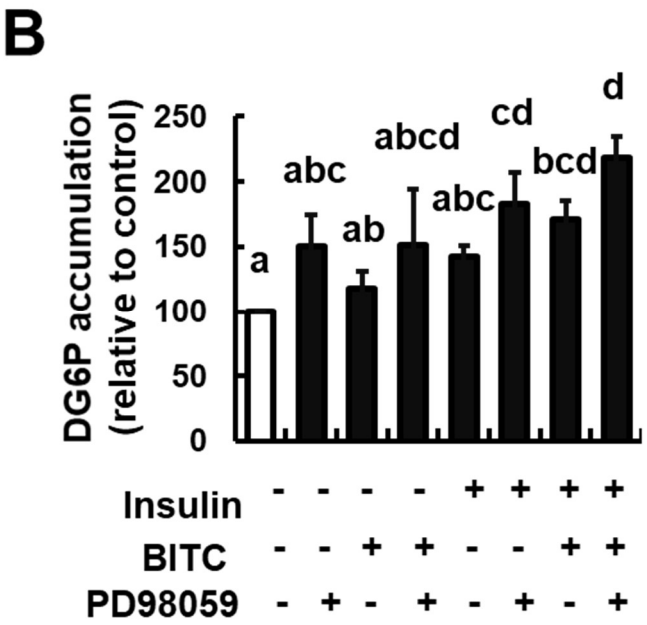


Figure 3.6 PD98059 inhibited BITC-induced ERK phosphorylation, but not glucose uptake. The confluent 3T3-L1 adipocytes were incubated with the differentiation-inducing media for 3 days, then starvation for 4 h. Thereafter, 20 μM PD98059 pretreated 1 h and treatment with 5 μM BITC for 30 min for the phosphorylated ERK (A) or glucose uptake (B), then the protein expression level was determined by Western blotting or the glucose uptake level was determined by an enzyme-dependent fluorometric assay. Data were analyzed by a one-way ANOVA followed by Tukey's HSD using XLSTAT software. Different letters above the bars indicate significant differences among treatments for each compound ($p < 0.05$).

3.5 Discussion

It has been reported BITC suppresses lipid droplet accumulation in 3T3-L1 preadipocytes (Kim et al., 2015) and improves the high-fat diet-induced liver fat accumulation in vivo (Alsanea et al., 2017). BITC effects on glucose uptake in mature 3T3-L1 adipocytes is unclear. Therefore, in this study we investigated the BITC effects on glucose uptake and the mechanism in mature 3T3-L1 adipocytes. In this study, we

demonstrated for the first time that BITC increased both basal and insulin-stimulated glucose uptake in mature 3T3-L1 adipocyte.

Our previous study showed that BITC suppresses lipid accumulation in 3T3-L1 preadipocytes during adipocyte differentiation. In this study, we demonstrated that BITC increases glucose uptake and lipid accumulation in the mature 3T3-L1 adipocytes. This discrepancy is might be due to differences in exposure time. In this study, after the confluent 3T3-L1 preadipocytes were incubated with differentiation-inducing medium for 3 days then exposure with BITC. However, in previously study, the confluent 3T3-L1 preadipocytes were incubated with the differentiation-inducing medium and exposure with BITC for 3 days. Moreover, some other reagents also show such effect on 3T3-L1 cells, such as 4-Hydroxyderricin (4HD) which is chalcone extracted from Angelica keiskei. In Zhang' s study, 4HD suppresses lipid accumulation during the differentiation-inducing phase and inhibits the differentiation of preadipocytes to adipocytes. However, in Li's study, they found that 4HD increases glucose uptake and lipid accumulation during the differentiation-maintaining phase and promotes adipogenesis in 3T3-L1 adipocytes.

The binding of insulin to cell surface receptor activates PI3K and phosphorylation of Akt at the plasma membrane. Akt activation enhances glucose uptake and adipogenesis gene expression in adipocyte tissue (Porstmann et al., 2009). Akt phosphorylation can enhanced 3T3-L1 adipocyte glucose uptake (Hu et al., 2014), while PI3K inhibitor, LY294002 inhibited glucose uptake (Yamamoto et al., 2010). In this study, BITC significantly increased phosphorylation of PI3K and Akt expression without affecting total protein expression. in mature adipocytes (Figs.3.3A and 3.3B). Additionally, PI3K inhibitor, LY294002, inhibited both basal and insulin-stimulated Akt phosphorylation (Figs.3.4Aand 3.4B) LY294002 inhibited BITC-induced glucose uptake (Fig.3.4C). It suggested that the BITC-stimulated adipogenesis pathway is

triggered by insulin-dependent improvement of PI3K/Akt pathway. MAPK cascades transduction by insulin is another signaling pathway involved in glucose uptake in cells in which ERK is a curial protein (Mokbel et al., 2014). It has been provided strong evidence indicated that enhancement of phosphorylated ERK1/2 promotes glucose uptake and GLUT4 translocation (Kayali et al., 2000). Our study showed that BITC significantly enhanced phosphorylated ERK1/2 and JNK1/2 (Figs.3.5A and 3.5B) and PD98059 inhibited BITC-induced phosphorylation of ERK (Fig.3.6A), while PD98059 could not inhibit BITC-induced glucose uptake (Fig.3.6B). Therefore, we can consider that BITC induced glucose uptake and lipid accumulation in mature 3T3-L1 adipocytes are involved in activation PI3K/Akt signaling pathway.

In conclusion, Akt is a key role in maintaining energy homeostasis in response to metabolic stress. In this study, we demonstrated for the first time that BITC increased glucose uptake through the PI3K/Akt signaling pathway in 3T3-L1 adipocytes. Further investigation of the events occurring with Akt activation, and in other transduction pathways, will be required to gain a more complete understanding of the mechanism of BITC-induced glucose uptake.

SUMMARY

Obesity and obesity-induced insulin resistance increase the risk of the major diseases closely related to metabolic syndrome. Adipose tissue, a highly insulin-responsive tissue, plays an important role in the regulation of glucose and lipid metabolism, insulin action, energy balance and inflammation. Benzyl isothiocyanate (BITC), an aromatic isothiocyanate compound, has been demonstrated to restrain the high-fat diet-induced body weight gain and liver fat accumulation. In this study, I have focused on the molecular mechanisms involved in the glucose and lipid metabolism influenced by BITC.

In the Chapter 1, the inhibitory effects of BITC on lipid accumulation in 3T3-L1 preadipocytes during differentiation-inducing was investigated. BITC inhibits lipid accumulation and glucose uptake in 3T3-L1 preadipocytes, possibly through inhibition of the adipocyte differentiation with the decreased expression of the adipogenic genes.

In the Chapter 2, I investigated whether BITC can ameliorate insulin resistance in 3T3-L1 mature adipocytes. BITC improves the insulin resistance in the mature 3T3-L1 adipocytes, possibly through the enhanced glucose uptake with the activated PI3K/Akt pathway.

In conclusion, the present study indicated that BITC is one of the potential food phytochemicals for prevention against not only obesity, but also obesity-related chronic diseases, such as insulin resistance-induced type 2 diabetes.

ACKNOWLEDGEMENTS

I am extremely grateful and appreciated that Professor Yoshimasa Nakamura provided me the opportunity to study as a doctoral student in his lab. I would like to express the deepest appreciation to him for his professional guidance, valuable suggestion and encouragement. If not for his guidance and help, I would not have been possible to finish this study.

I would like to thank Professor Yoshiyuki Murata for his academic support and suggestion. Thanks go to Shintaro Munemasa and Toshiyuki Nakamura for their precious advice.

Special thanks go to Ikumi Sasaki for her academic support. It is my pressure to pressure to thank to Zhe Liu, Yujia Liu, Xiaoyang Liu, Wensi Xu and Qifu Yang, as well as other students in this lab. Their company and supporting makes me feel less homesick.

The scholarship from China Scholarship Council financially supported me during entire doctoral period.

Finally, I would like to give my sincere thanks to my family and friends for their patient and support.

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