A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Iberin inhibits adipogenesisin 3T3-L1 preadipocytes by suppressing Akt signaling

이베린의Akt 신호전달과정 억제를 통한 지방세포분화 저해 효능

By

Yu, Jae Gak

Department of Agricultural Biotechnology

Seoul National University

August, 2013

석사학위논문

Iberin inhibits adipogenesis in 3T3-L1 preadipocytes by suppressing Akt signaling

이베린의Akt 신호전달과정 억제를 통한 지방세포분화 저해 효능

지도교수 이 형 주

이 논문을 석사학위 논문으로 제출함

2013년 8월

서울대학교 대학원
농생명공학부
유 재 각

유재각의 석사학위논문을 인준함
2013년 8월

위 원 장 강 동 현 (인)

부위원장 이 형 주 (인)

위 원 이 기 원 (인)

Abstract

Iberin is a naturally derived isothiocyanate in cruciferous vegetables such as savoy cabbage, cauliflower, and broccoli. It has been reported to exhibit anti-cancer effects. However, anti-obesity effect and its molecular mechanism have not been elucidated. Herein, I examined the anti-adipogenic effect of iberin and its associated mechanisms in 3T3-L1 preadipocytes. Iberin effectively inhibited MDI (Methyl isobutylxanthine, Dexamethasone, Insulin)-induced adipogenesis in the early stage of adipocyte differentiation and the expression of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα) which are key regulators of adipogenesis and lipid accumulation. Fluorescein-activated cell sorter (FACS) analysis data showed that iberin suppressed MDI-induced cell cycle progression, mitotic clonal expansion (MCE). Western blotanalysis demonstrated that not extracellular-signal-regulated kinase (ERK) but Akt signaling was attenuated by iberin. Collectively, these finding suggest thatiberin possesses anti-adipogenic activity by suppressing Akt signaling pathway.

Key Words: Iberin; Akt signaling; adipogenesis; 3T3-L1 preadipocytes

Student ID: 2011-24115

Contents

Ⅰ. Introduction

Increasing number of obesity is a serious social problem because obesity is associated with clinical complications such as type 2 diabetes, hypertension, cardiovascular diseases, and several types of cancer [1, 2]. Therefore, a number of researchers have developed therapeutic strategies for preventing or treating obesity [3-5]. The cause of obesity and overweight is an energy imbalance between food intake and energy expenditure [6]. Excess energy intake relative to energy consumption induces expansion of fat mass through increasing adipocyte volumes (hypertrophy) and adipocyte numbers (hyperplasia). Hyperplasic growth is related to preadipocyte differentiation at early stages and hypertrophy is induced by lipid accumulation in mature adipocytes [7, 8]. Therefore, the modulation of adipose tissue mass by inhibiting adipogenesisis one of attractive strategies for the prevention of obesity [9, 10].

Adipogenesis consists of two stages which are controlled by a cascade of several transcription factors and signaling pathway during adipocyte differentiation [11-13]. At early stage, growth-arrested preadipocytes reenter the cell cycle that is mitotic clonal expansion (MCE) which is a prerequisite for adipogenesis [14]. Mitotic clonal expansion is regulated by a variety of signaling pathway which is involved in cell

proliferation and expression of CCAAT/enhancer-binding proteinβ (C/EBPβ) [15]. Particularly, adipocyte differentiation is controlled by mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and Phosphatidylinositide 3-kinase (PI3K)/Protein kinase B (PKB or Akt)/Mammalian target of rapamycin (mTOR) signaling pathways [16-18]. In terminal differentiation, peroxisome proliferator-activated receptor γ(PPARγ) and CEBPα play roles of master regulator proteins of mature adipocyte differentiation by activating each other [19-21].

Cruciferous vegetables contain various isothiocyanates such as sulforaphane, phenethylisothiocyanate (PEITC). Many previous studies have shown that isothiocyanates suppress carcinogenesis, cardiovascular and neurological diseases in various animal models [22, 23]. Iberin, one of isothiocyanates, is natural compounds found in cruciferous vegetables including cabbage, cauliflower, broccoli, and kale [24]. In the previous studies, it was reported that iberin inhibited growth and induced apoptosis in several types of cancer cell, and it was natural quorum sensing inhibitor of *Pseudomonas aeruginosa* [25-28]. However, there was no report about the effect of iberin on obesity or adipogenesis.

Herein, I demonstrate that iberin inhibits adipogenesis and intracellular lipid accumulation in 3T3-L1 preadipocytes. Iberin was found to suppress cell cycle progression in 3T3-L1 preadipocytes, thereby

suppressing mitotic clonal expansion. Iberin also reduced MDI-induced activation of Akt signaling, but not ERK signaling. Collectively, my results show that iberin effectively inhibits adipogenesis and lipid accumulation by suppressing Akt signaling pathway.

Ⅱ.Materials and Methods

2.1. Materials

Iberin was purchased from LKT laboratories, Inc. (St. Paul, MN). Dulbecco's Modified Eagle medium (DMEM), fetal bovine serum (FBS), bovine serum (calf serum), Antibiotic-Antimycotic were purchased from Gibco®, Life Technologies Corporation (Grand Island, NY). Antibodies against C/EBPα, phospho-MEK1/2 (Ser217/221), phospho-p90RSK (Thr359/363), phospho-Akt (Thr308), phospho-GSK-3β (Ser9), phospho-p70 S6 Kinase (Thr389), phospho-S6 Ribosomal Protein (Ser235/236), total MEK1/2, total RSK1/RSK2/RSK3, total Akt, total GSK-3β, total p70 S6 Kinase, total S6 Ribosomal Protein were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Also, antibodies against PPARγ, Cyclin D1, Cyclin A, phospho-ERK, total ERK1, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody against phospho-Akt (Ser473) was from GenScript USA, Inc. (Piscataway, NJ). Antibody against β-actin, 3-Isobutyl-1-methylxanthine (IBMX), insulin, dexamethasone, and Oil Red O was obtained from Sigma-Aldrich Co. LLC (St. Louis, MO). Isopropyl alcohol was purchased from AMRESCOLLC (Solon, OH). PI3 Kinase (p110α/p85α) active protein, PDK1 active protein, and PDKtidewere

obtained from Millipore (Billerica, MA) and phosphatidylinositol was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL).

2.2. Cell culture and preadipocytes differentiation

3T3-L1 cell line was obtained from ATCC (Manassas, VA). All cell culture materials were purchased from Gibco®, Life Technologies Corporation (Grand Island, NY). 3T3-L1 preadipocytes were cultivated in 10% bovine serum (calf serum)/Dulbecco's Modified Eagle medium (DMEM) at 5% CO_2 and 37°C until confluence. Confluent cells were stimulated with MDI induction media (10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone, 5 μg/ml insulin in DMEM) for 2 days. And then, the media were changed to Insulin media (10% FBS, 5 μg/ml insulin in DMEM) for 2 days. The cells were cultured in 10% FBS/DMEM for 2 days. Finally, 3T3-L1 preadipocytes fully differentiated to adipocytes.

2.3. Oil Red O staining

3T3-L1 preadipocytes (2.5×10^4 cells per well) were seeded in 24 well plate. After confluence, the cells were incubated with differentiation conditions (described above) for 6 days. The media were removed and the differentiated 3T3-L1 adipocytes were fixed with 4% formaldehyde solution

for 20 min. And then, formaldehyde solution was removed and the fixed cells were washed with phosphate buffered saline(PBS). Next, the cells were stained with Oil Red O solution (0.2 g of Oil Red O powder in 40 ml of 60% isopropyl alcohol and filtering by 0.45 μm filter membrane; Nalgene®, Thermo Fisher Scientific Inc., Waltham, MA) for 15 min. Next, the stained cells were washed with PBS twice. Finally, stained Oil Red O was eluted with isopropyl alcohol and theamount of this was measured with spectrophotometry at 515 nm.

2.4. MTS assay

3T3-L1 preadipocytes (1.5×10^4 cells per well) were seeded in 48 well plate, and then incubated until confluence. After that, the cells were treated with 10% FBS/DMEM which contains 5, 10, 20, 40, 80 μM of iberin or only 10% FBS/DMEM as a control. Viability of the cells was measured using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation,Fitchburg, WI), which is composed of is composed of solutions of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) andphenazinemethosulfate (PMS).After culturing for 72 hours, 30 μl of MTS/PMS solution (ratio 1 ml/50 μl) were added to each well, and the cells wereincubated for 1 h at 5% CO_2 and 37°C. After that, the plate was measured with a microplate reader

at 490 nm.

2.5. Western blot assays

3T3-L1 preadipocytes (1.5×10^5 cells per dish) were seeded in 6cm dishes and then incubated until confluence. After that, the media were changed to MDI induction media with or without 5, 10, 20 μM of iberin. Control is changed to 10%FBS/DMEM. The cell lysates were centrifuged at 10 min, 14,000 rpm, 4°C and supernatants were collected. The protein concentration of supernatants was measured using *DC*™ Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA) as described in the manufacturer's manual. Same amount of protein was loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. The loaded gels were transferred to BioTrace™ polyvinylidene fluoride (PVDF) Transfer Membrane (PALL Corporation,Port Washington, NY). The membranes were blocked with skim milk blocking buffer. Subsequently, the membranes were incubated with specific primary antibodies, and then incubated with HRP-conjugated secondary antibodies. The protein bands of membranes were visualized using chemiluminescent labeling detection reagents (GE Healthcare Life Sciences, Little Chalfont, UK).

2.6. Quantitative real time polymerase chain reaction (qPCR)

3T3-L1 preadipocytes were seeded in 6cm dishes as described above. Total RNA was isolated using RNA-Bee reagent (Tel-Test, Inc., Friendswood, TX) as described in the manufacturer's manual. The concentration of RNA was quantified using NanoDrop™ ND-2000 (Thermo Fisher Scientific Inc., Waltham, MA). cDNA was synthesized using a reverse transcription system obtained from Promega Corporation (Fitchburg, WI). For qPCR, cDNA and primers was added to a Maxima® SYBR Green qPCRMaster Mix (Fermentas,Vilnius, LT). The primer sequences were as follows: PPARγ (NM_011146), 5'-CGCTGATGCACTGCCTATGA-3' and 5'-AGAGGTCCACAGAGCTGATTCC-3'; C/EBPα (BC058161), 5'-CGCAAGAGCCGAGATAAAGC-3' and 5'-CACGGCTCAGCTGTTCCA-3'; aP2 (NM_024406), 5'-CATGGCCAAGCCCAACAT-3' and 5'-CGCCCAGTTTGAAGGAAATC-3'; Fatty acid synthase (FAS, NM_007988), 5'-CTGAGATCCCAGCACTTCTTGA-3' and 5'-GCCTCCGAAGCCAAATGAG-3'; β-actin (X03672), 5'-TGTCCACCTTCCAGCAGATGT-3' and 5'-AGCTCAGTAACAGTCCGCCTAGA-3'. All primers were obtained from Bioneer Corporation (Daejeon, Republic of Korea).

2.7. Flow cytometry using a fluorescence-activated cell sorter (FACS)

3T3-L1 preadipocytes (1.0×10^5 cells per dish) were seeded in 3cm dishes and then incubated until confluence. After that, the media were changed to MDI induction media with or without 20 μM of iberin. Control is changed to 10%FBS/DMEM. The cells were dissociated by trypsin and centrifuged at 2000 rpm, 4 °Cfor 2 min. The supernatants were removed and the pellets were re-suspended in PBS, and then centrifuged at 2000 rpm, 4 °C for 2 min again. The pellets were fixed by suspension in cold 70% (v/v) ethanol and maintained at -20 °C overnight. And then, the fixed cells were centrifuged at 1500 rpm, 4 °C for 3 min. After that, the pellets were re-suspended in 600μl of PBS containing 20 μg/ml of PI solution (Sigma-Aldrich Co. LLC, St. Louis, MO) and 0.2 mg/ml of RNase (AMRESCO LLC, Solon, OH). Next, the cells were incubated at 37 °C for 15 min.Lastly, the fluorescence-activated cells were measured using guava easyCyte™ Flow Cytometer (Millipore, Billerica, MA).Ten thousand cells per each sample were analyzed [29].

2.8. PI3K kinase assay

PI3K active protein (100 ng) was incubated with iberin or LY294002 at 30 °C for 10 min. And then, phosphatidylinositol was added to the mixtures and incubated at room temperature for 5 min, followed by incubation with the reaction buffer (100 mM HEPES pH 7.6, 50 mM $MgCl_2$, 250 μM ATP including 10 μCi of [γ-^{32}P]ATP) at 30 °C for 10min. The reaction was stopped by adding 15 μl of 4N HCl and 130 μl of chloroform and methanol mixture (1:1). To separate phases, the mixtures were mixed vigorously for 30 sec. After that, 30 μl of the separated lower chloroform phase was spotted on thin layer chromatography (TLC) Silica gel 60 F_{254} (Merck Chemicals, Darmstadt, Germany), which was activated at 110 °C for 1 h previously. Finally, ^{32}P-labelled phosphatidylinositol phosphate (PIP) was separated by TLC, and the amount of radio-labeled PIP were visualized using autoradiography.

2.9. PDK1 kinase assay

10X Reaction buffer (500 mMTris/HCl pH 7.5, 0.5% 2-mercaptoethanol) and PDK1 active protein (126ng) were mixed, andthe mixtures incubated with iberin or staurosporine at 30 °C for 10 min. And then, the volume of mixture was filled by dH_2O and PDKtide was added to

the mixtures, followed by incubation with [γ–^{32}P] ATP solution dilutedwith magnesium–ATP cocktail at 30 °C for 10 min.And then, aliquots were transferred onto p81 phosphocellulose paper (Millipore, Billerica, MA) and washed with 0.75% phosphoric acid three times and acetone once for 5min. The radioactive incorporationwas determined using a scintillation counter.

2.10. Statistical analysis

When necessary, data were expressed as means ± S.D. values, and the Student's t-testwas used for single statistical comparisons. A probability value of $p < 0.05$, $p < 0.01$ was used as the criterion for statistical significance.All analyses were performedusing statistical analysis software.

Ⅲ.Results

3.1. Iberin inhibits MDI-induced adipogenesis in 3T3-L1 preadipocytes

To investigate anti-adipogenic effect of iberin in 3T3-L1 preadipocytes, adipogenesis was assessed by Oil Red O staining of intracellular lipid droplets. The adipocyteswere differentiatedaccording to Materials and Methods as described above. Relative lipid contents of MDI-treated cells were increased by 3-fold compared to undifferentiated cells. However, treatment of iberin effectively suppressed MDI-induced intracellular lipid accumulation at 5, 10, 20 μM in a dose-dependent manner (Fig. 1A). Microscope images and scanned images of stained dishes showed consistent results (Fig. 1B). To prove that the inhibition of lipid accumulation is not owing to induction of cell death by iberin, I measured cell viability using MTS assay. Iberin did not significantly affect cell viability up to 20 μM of iberin (Fig. 1C). Taken together, iberin suppresses MDI-induced adipogenesis and intracellular lipid accumulation in 3T3-L1 preadipocytes.

3.2. Iberin suppresses MDI-induced expression of adipogenic markers in 3T3-L1 preadipocytes

PPARγ and C/EBPα are two master regulators of adipogenesis [20]. To identify the effect of iberin on expression of these proteins, I performed western blot analysis. The western blot results showed that iberin considerably inhibited MDI-induced the expression levels of PPARγ and C/EBPα proteins (Fig. 2A). mRNA expression levels of PPARγ, C/EBPα, and their down-stream genes, aP2 and fatty acid synthase (FAS) were also measured. Iberin suppressed relative mRNA expression of PPARγ, C/EBPα, aP2, and FAS (Fig. 2B). Therefore, iberin inhibits adipocyte differentiation by suppressing master regulators of adipogenesis.

3.3. Iberin suppresses Mitotic Clonal Expansion (MCE), early stage of adipogenesis in 3T3-L1 preadipocytes

Adipogenesis consists of two steps including mitotic clonal expansion and terminal differentiation, respectively [30]. Because iberin suppressed adipogenesis, I confirmed that iberin effectively acts on which step of adipogenesis. I treated 20 μM of iberin at different period of adipogenesis during 6 days (Fig.3A). Oil Red O staining data showed that iberin inhibited adipocyte differentiation significantly when it was treated

during the 0-2 days. Iberin reduced adipogenesis effectively when it was treated on the duration including 0-2days (Fig. 3B, 3C). It suggests that iberin inhibits MDI-induced adipogenesis by suppressing mitotic clonal expansion.

3.4. Iberin represses MDI-induced cell cycle progression in 3T3-L1 preadipocytes

Growth-arrested 3T3-L1 preadipocytes reenter cell cycle and undergo mitotic clonal expansion [14]. The cell cycle progression were measuredby FACS analysis. FACS results showed that MDI cocktail induced cell cycle progression at 16, 20, and 24 h (Fig. 4A). However, iberin repressed MDI-induced cell cycle progression by inducing G_1 arrested state (Fig. 4A, 4B). Western blot analysis showed that iberin inhibited the protein expression of cyclin D1 and cyclin A, which control the progression of cell cycle by activating cyclin-dependent kinase (Cdk) enzymes at G_1/S phase transition [31, 32]. Therefore, iberin suppressed cell cycle progression by inducing G_1 arrested state and consequent on the suppression of cell proliferation in 3T3-L1 preadipocytes.

3.5. Iberin inhibits MDI-induced Akt signaling but not ERK

signaling in 3T3-L1 preadipocytes

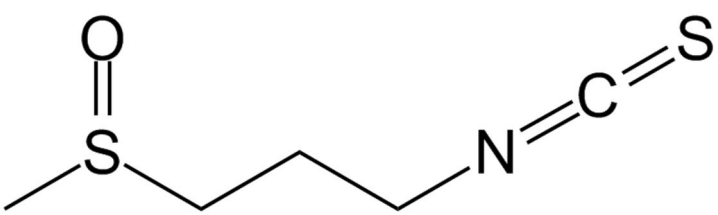
PI3K/Akt and ERK signaling pathways are well-known to regulatecell proliferation in various studies [33]. Both pathways play necessary roles mitotic clonal expansion in adipogenesis turning on by insulin as a growth factor [34]. First of all, I investigated ERK signaling such as MEK, ERK, and RSK. Iberin did not inhibit the phosphorylation of MEK, ERK, and RSK proteins (Fig. 5A). Secondly, I examined the effects of iberin on Akt signaling pathway as a downstream signaling of PI3K. As a result, iberin suppressed the phosphorylation of Akt (Ser473 and Thr308). Phosphorylation of GSK-3β, p70S6K and S6, the downstream regulators of Akt, were also decreased (Fig. 5B). These observations suggested that iberin suppressed adipogenesis by inhibiting Akt signaling pathway.

3.6. Iberin does not affect PI3K and PDK1 activities

To investigate how iberin regulate Akt phosphorylation, I measuredPI3K and phosphoinositide-dependent kinase 1 (PDK1) activities using *in vitro*kinase assay. Especially, Thr308 of Akt was phosphorylated by PDK1 [35]. Iberin did not affect PI3K activity compared to LY294002, PI3K inhibitor (Fig. 6A). Also, PDK1 activity was not reduced by iberin compared to staurosporine, ATP-competitive kinase inhibitor (Fig. 6B). Therefore, iberin inhibited Akt signaling, but did not affect PI3K and PDK1

activities.

Figure 1



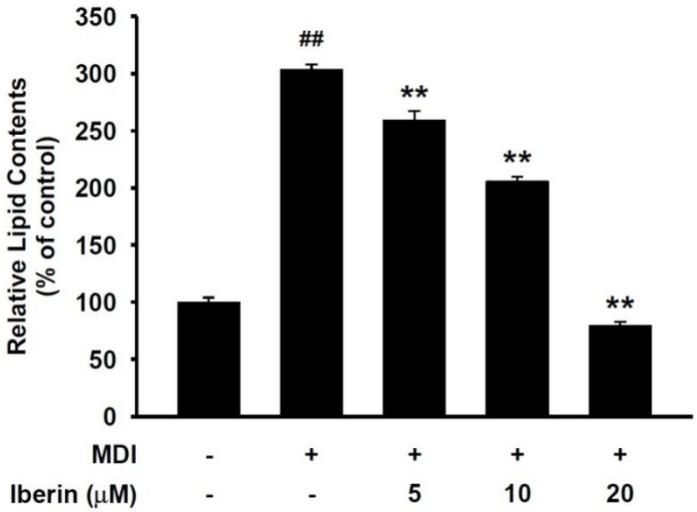
Iberin

Figure 1. The structure of Iberin

Structure of iberin, one of the isothiocyanates.

Figure 2

A


Relative Lipid Contents (% of control)
350
300
250
200
150
100
50
0
##
**
**
**
MDI - + + + +
Iberin (μM) - - 5 10 20

B

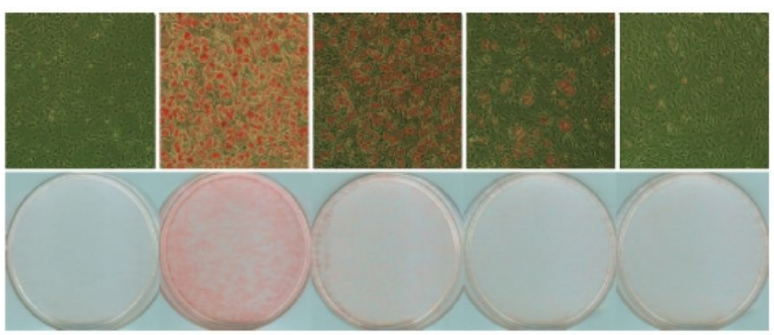

MDI - + + + +
Iberin (μM) - - 5 10 20

Figure 2

C

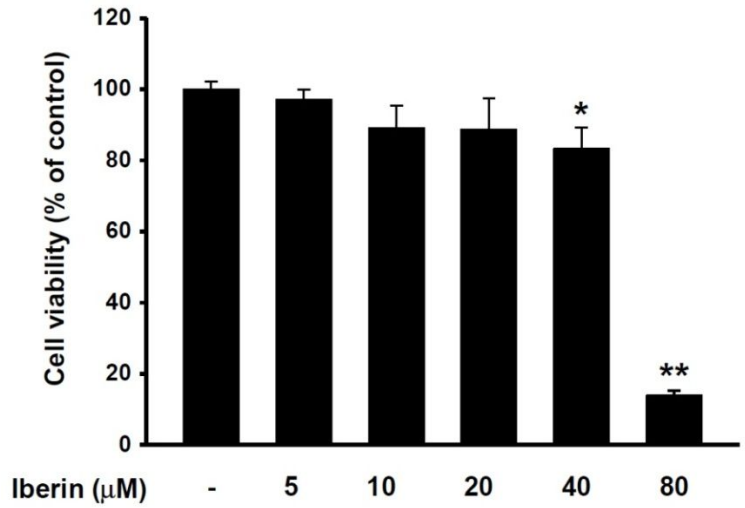
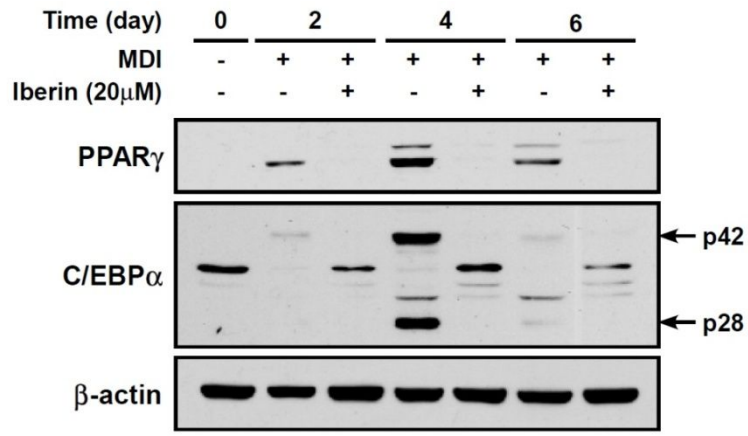

Cell viability (% of control)
120
100
80
60
40
20
0
*
**
Iberin (μM)
-
5
10
20
40
80

Figure 2. Effect of iberin on MDI-induced adipogenesis in 3T3-L1 preadipocytes

(A) Quantitative Oil Red O staining data and (B) photographs of Oil Red O staining show that intracellular lipid accumulation is effectively suppressed by 20 μM of iberin in 3T3-L1 preadipocytes. (C) Iberin does not affect cell viability, up to 20 μM concentration. 3T3-L1 preadipocytes. After reaching 100% confluency of 3T3-L1 preadipocytes, the cells were treated with iberin for 72 h. Data are representative of 3 independent experiments that shows similar results. Data are presented as a percent of control and are expressed as means ± S.D. Significant differences between the control and the MDI-treated group (##, $P < 0.01$).Significant differences between the group treated with MDI only and the group treated with MDI and iberin (**, $P < 0.01$ and *, $P < 0.05$, respectively).

Figure 3

A


Time (day)
0
2
4
6
MDI
Iberin (20μM)
PPARγ
C/EBPα
p42
p28
β-actin

B

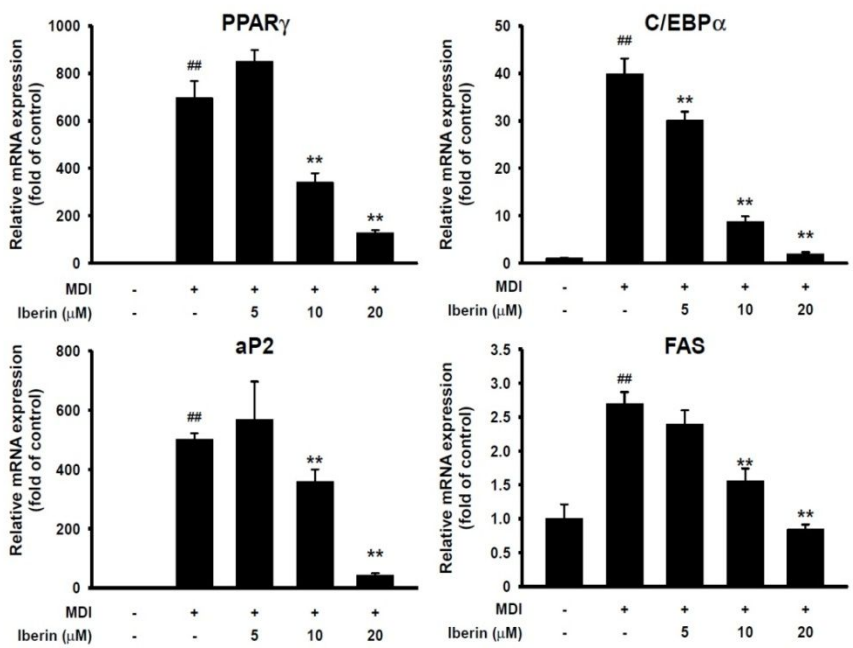
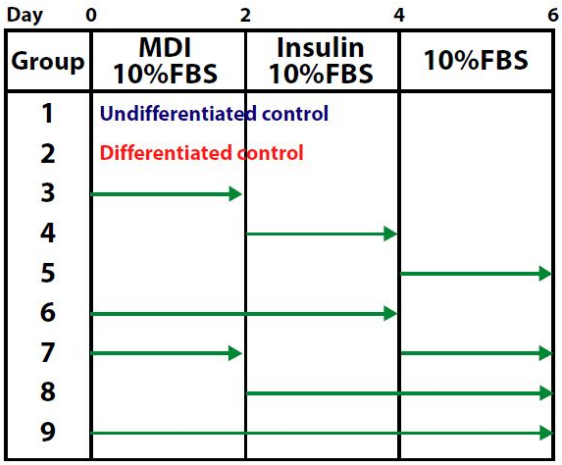

PPARγ
C/EBPα
aP2
FAS
Relative mRNA expression (fold of control)
MDI
Iberin (μM)
##
**

Figure 3. Effect of iberin on MDI-induced adipogenic markers in 3T3-L1 preadipocytes

(A) Iberin inhibited MDI-induced PPARγ and C/EBPα expression, determined by western blot assay. Arrows marked on the band of C/EBPα point to specific C/EBPα proteins (B)Iberin suppressed the mRNA expressions of PPARγ, C/EBPα and downstream genes, such as aP2 and FAS at 6 days, as determined by real-time RT-PCR and normalized to β-actin mRNA levels.Data are representative of 3 independent experiments that shows similar results. Data are presented as a percent of control and are expressed as means ± S.D. Significant differences between the control and the MDI-treated group (##, $P < 0.01$).Significant differences between the group treated with MDI only and the group treated with MDI and iberin (**, $P < 0.01$).

Figure 4

A


Day 0 2 4 6
Group
MDI 10%FBS
Insulin 10%FBS
10%FBS
1
2
3
4
5
6
7
8
9
Undifferentiated control
Differentiated control

B

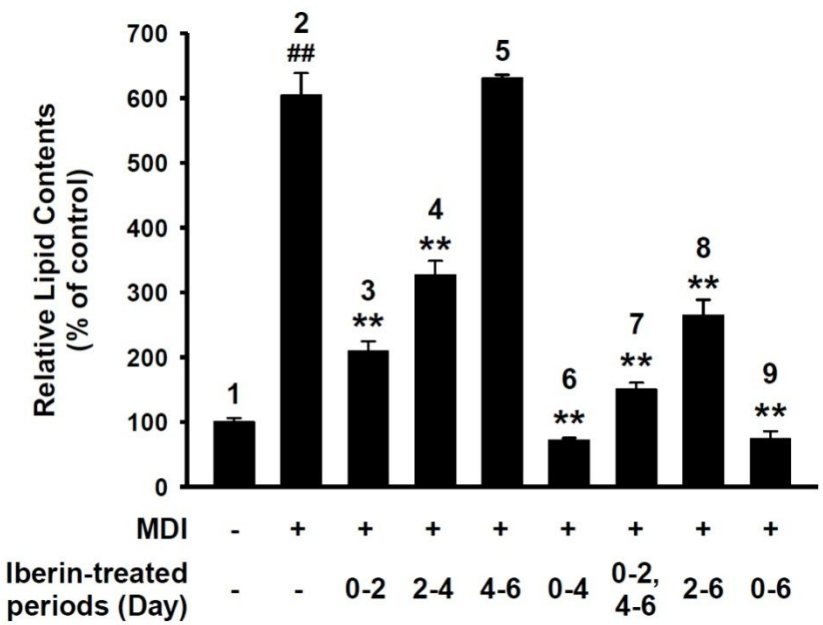

Relative Lipid Contents (% of control)
700
600
500
400
300
200
100
0
1
2 ##
3 **
4 **
5
6 **
7 **
8 **
9 **
MDI - + + + + + + + +
Iberin-treated periods (Day) - - 0-2 2-4 4-6 0-4 0-2, 4-6 2-6 0-6

Figure 4

C

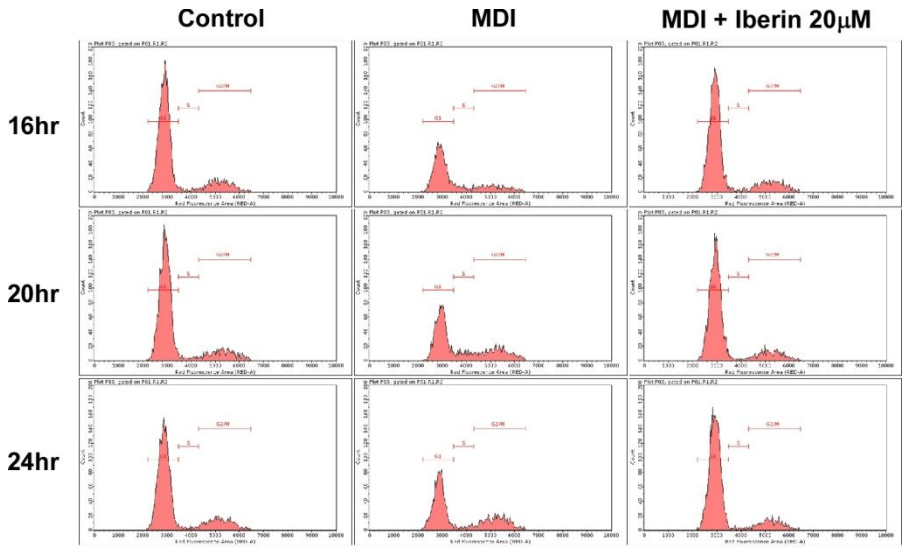
MDI	-	+	+	+	+	+	+	+	+
Iberin-treated periods (Day)	-	-	0-2	2-4	4-6	0-4	0-2, 4-6	2-6	0-6

Figure 4. Effect of iberin on the early stage of MDI-induced adipogenesis in 3T3-L1 preadipocytes

(A) A schematic diagram of experiment; time schedule for duration treated iberin for each group. Iberin was treated at different times for a total of 6 days during cell differentiation. (B) Oil Red O staining data and (C) photographs of Oil red O staining show that the inhibitory effect of iberin mainly occurs when treated in the first two days (0-2 days).Data are representative of 3 independent experiments that shows similar results. Data are presented as a percent of control and are expressed as means ± S.D. Significant differences between the control and the MDI-treated group (##, $P < 0.01$).Significant differences between the group treated with MDI only and the group treated with MDI and iberin (**, $P < 0.01$).

Figure 5

A


Control
MDI
MDI + Iberin 20μM
16hr
20hr
24hr

B

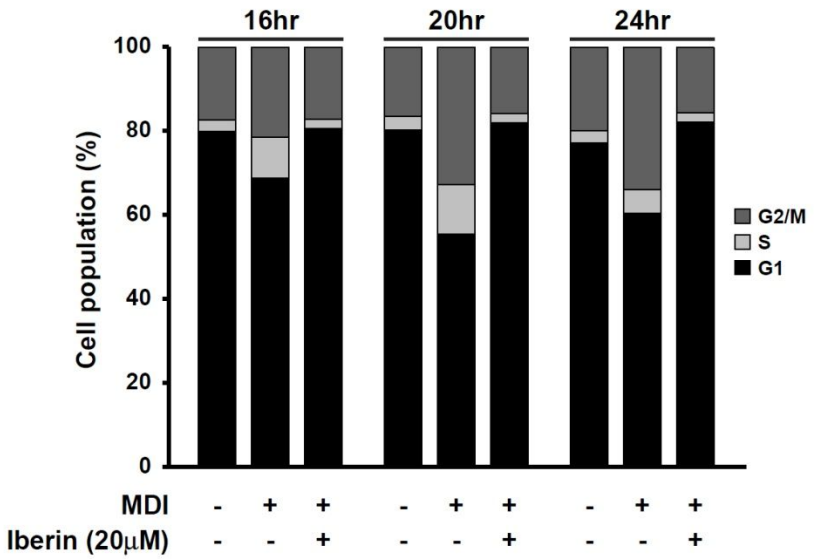

16hr
20hr
24hr
100
80
60
40
20
0
Cell population (%)
G2/M
S
G1
MDI - + + - + + - + +
Iberin (20μM) - - + - - + - - +

Figure 5

C

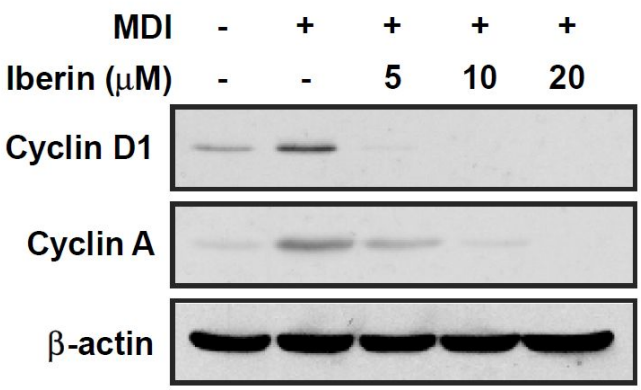


Figure 5. Effect of iberin on MDI-induced cell cycle progression in 3T3-L1 preadipocytes

(A) FACS analysis is performed at 16, 20, 24 h after MDI only or MDI with iberin treatment. As shown in FACS analysis data, MDI induced cell cycle progression but, iberin suppressed the MDI-induced cell cycle progression by inducing G_1 arrested state. (B) The population of cells in each stage of the cellcycle was quantified. (C) Iberin suppressed expression of cyclin D1 and cyclin A in 3T3-L1 preadipocytes, as determined by western blotting. Data are representative of 3 independent experiments that shows similar results.

Figure 6

A

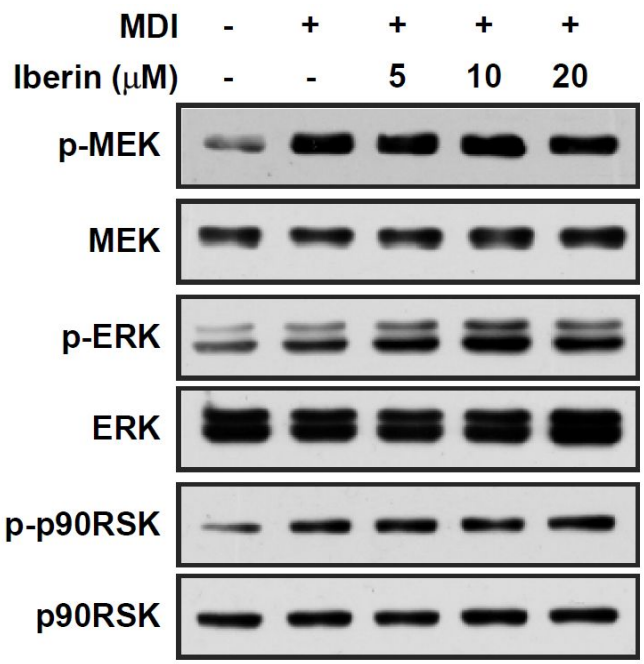


Figure 6

B

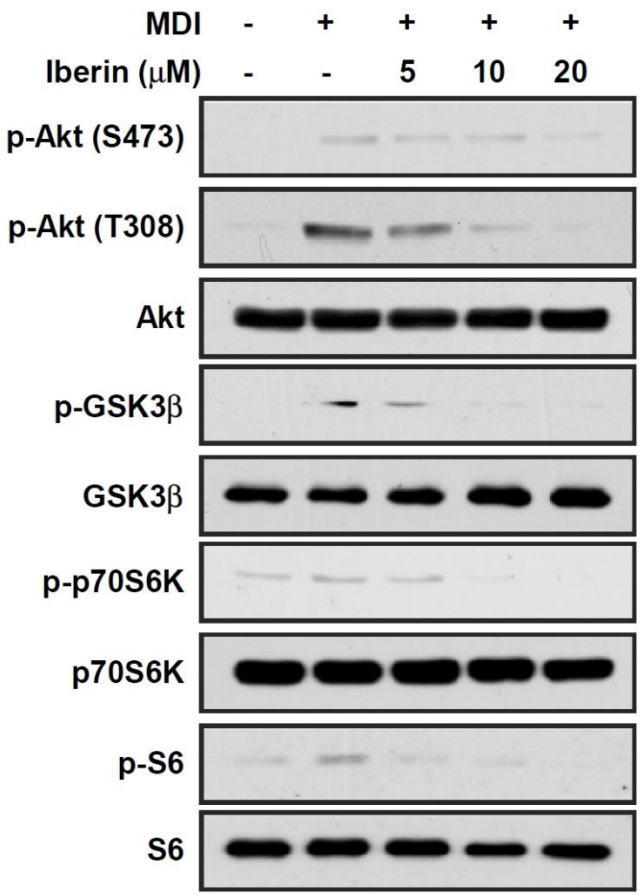
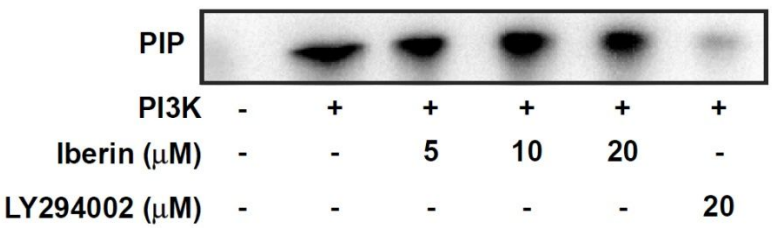

MDI - + + + +
Iberin (μM) - - 5 10 20
p-Akt (S473)
p-Akt (T308)
Akt
p-GSK3β
GSK3β
p-p70S6K
p70S6K
p-S6
S6

Figure 6. Effect of iberin on MDI-induced ERK and Akt signaling pathways in 3T3-L1 preadipocytes

(A) Iberin did not suppress the phosphorylation of MEK, ERK, p90RSK. However, (B) the phosphorylations of Akt signaling including GSK-3β, p70S6K, S6 were decreased by iberin, as determined by western blot assay. Data are representative of 3 independent experiments that shows similar results.

Figure 7

A


PIP
PI3K - + + + + +
Iberin (μM) - - 5 10 20 -
LY294002 (μM) - - - - - 20

B

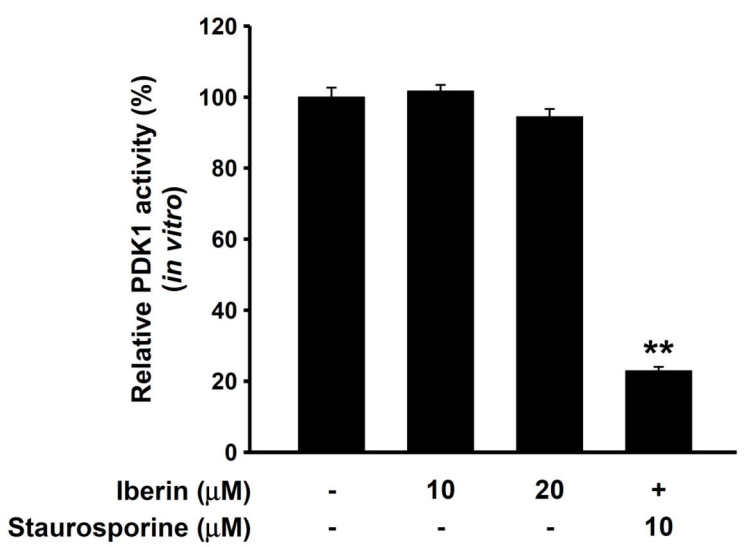

Relative PDK1 activity (%)
(in vitro)
120
100
80
60
40
20
0
**
Iberin (μM) - 10 20 +
Staurosporine (μM) - - - 10

Figure 7. Effect of iberin on PI3K and PDK1 activities in 3T3-L1 preadipocytes

(A) Iberin did not inhibit active PI3K activity CPE inhibited active IR kinase activity*in vitro*. (B) Also, *in vitro*active PDK1 activity was not suppressed by iberin. *In vitro* kinase assay was performed as described in Materials and Methods. The results were determined from three independent experiments. The data arepresented as a percent of control, expressed as means±S.D.Significant differences between the active kinaseincubated without and with samples (**, $P<0.01$).

Figure 8

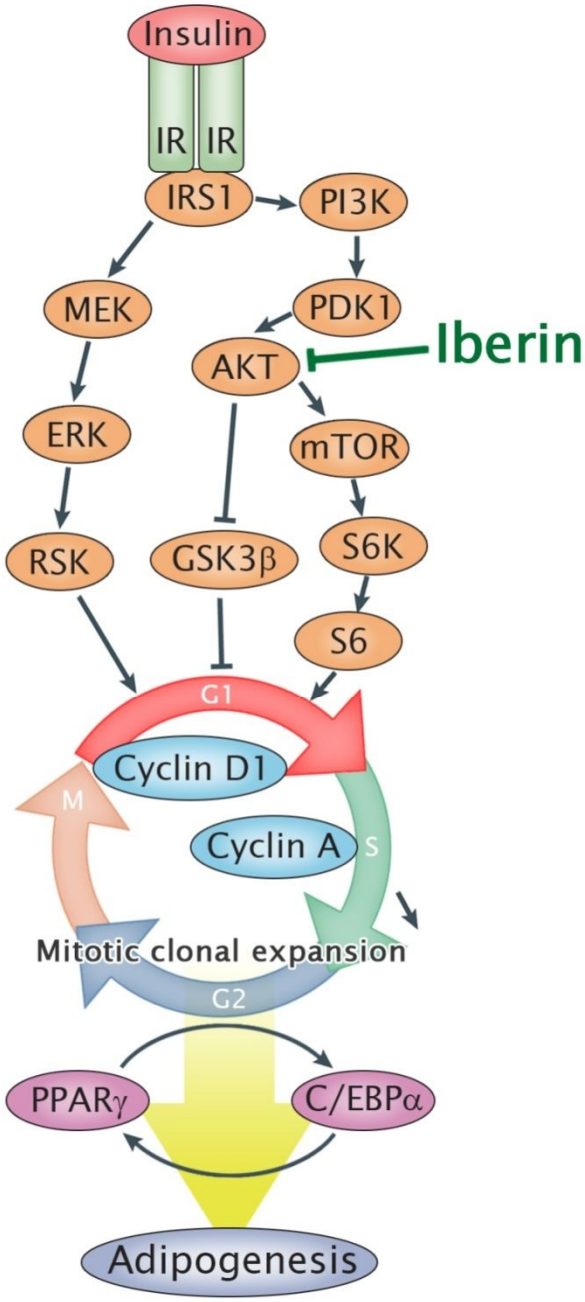

Insulin
IR
IR
IRS1
PI3K
MEK
PDK1
Iberin
AKT
ERK
mTOR
RSK
GSK3β
S6K
S6
G1
Cyclin D1
M
Cyclin A
S
Mitotic clonal expansion
G2
PPARγ
C/EBPα
Adipogenesis

Figure 8. Hypothetical molecular mechanism of iberin in preventing adipogenesis in 3T3-L1 preadipocytes

Iberin inhibits adipogenesis of 3T3-L1 preadipocytes by suppressing Akt signaling.

Ⅳ. Discussion

In the present study, I have demonstrated that iberin effectively inhibits adipogenesis and intracellular lipid accumulation in 3T3-L1 preadipocytes. Iberin greatly suppresses the expression of PPARγ and C/EBPα, two master regulators of adipogenesis. In further mechanism study, it had the best reducing effect of adipogenesis when it was treated at early stage of adipogenesis, mitotic clonal expansion. Mitotic clonal expansion is associated with cell cycle progression, so I confirmed that iberin effectively suppressed the cell cycle progression by inducing G_1 arrested state. Results showed that iberin had anti-adipogenic effect by suppressing Akt signaling.

Akt/mTOR signaling cascade can trigger multiple processes such as proliferation, apoptosis, differentiation, metabolism [36, 37]. According to my results, iberin effectively inhibits Akt and the downstream of Akt including GSK-3β, p70S6K, and S6. However, the upstream of Akt, such as PI3K and PDK1 was not affected by iberin. ERK pathway, insulin receptor (IR), and insulin receptor substrate 1 (IRS1) were not regulated by iberin. Thus, to identify the specific molecular mechanism of iberin, further study how iberin regulates Akt and molecular target of iberin remains to be elucidated.

Results showed that the presence of iberin over the 0-2 days of adipogenesis exhibits the best effect of the inhibition of adipocyte

differentiation. Since the first two days are associated with mitotic clonal expansion, I confirmed whether iberin inhibited the cell cycle progression and cell cycle regulated components. However, Fig. 4C also showed that iberin repressed adipogenesis during 2-6 days as well as 0-2 days. In the late stage of adipogenesis, PPARγ regulates lipogenesis by inducing transcription of lipogenic genes including FAS, aP2 [38, 39]. Also, sterol regulatory element binding protein 1 (SREBP-1), the regulator of lipogenesis, is regulated by Akt/mTOR signaling and mediates the expression of PPARγ [40, 41]. Furthermore, inhibition of lipogenesis induces thermogenesis, as increase of energy expenditure [42]. As lipolysis is other anti-obesity therapeutic target, the increase of lipolysis up-regulates fatty acid utilization and energy expenditure [43]. Moreover, upward thermogenesis in brown adipose tissue (BAT) effectively increases energy expenditure; it induces reduction of adipose tissue [44]. It is also possible that iberin mediates lipolysis and thermogenesis. Therefore, I should investigate anti-obesity effect of iberin through various approaches.

Iberin has yet not been investigated for the prevention of human diseases, whereas sulforaphane and PEITC, their natural compounds found in broccoli, sprouts and kale are well-characterized isothiocyanates with preventive effects against various human diseases [22]. Recent studies have shown that sulforaphane inhibits adipogenesis through cell cycle arrest [45].

It was suggested that sulforaphane inhibits the ERK signaling which is involved in cell proliferation, followed by cell cycle arrest and adipogenesis. However, iberin inhibits Akt signaling effectively but not ERK signaling.

Up-regulation of PTEN, tumor suppressor against PI3K, increases energy expenditure in mice [46]. Inhibition of Akt signaling induces insulin sensitivity and weight loss [47]. Furthermore, mTOR/S6K signaling is related with energy balance, obesity, and diabetes [48, 49]. Therefore, attenuating Akt signaling is an attractive target for obesity. These studies suggest that iberin has a possibility to inhibit obesity *in vivo*. Therefore, a high-fat diet-induced mouse model is needed for identification of anti-obesity effect of iberin.

In summary, present study shows that iberin significantly inhibits adipogenesis and intracellular lipid accumulation by suppressing Akt signaling. I also found that iberin inhibited Akt signaling and, thereby suppressing the mitotic clonal expansion. Collectively, my study suggests that iberin might be used as a phytochemical in the prevention of obesity.

Ⅴ. References

1. Kopelman, P.G., *Obesity as a medical problem.* Nature, 2000. **404**(6778): p. 635-43.
2. Cao, Y., *Adipose tissue angiogenesis as a therapeutic target for obesity and metabolic diseases.* Nat Rev Drug Discov, 2010. **9**(2): p. 107-15.
3. Valentino, M.A., J.E. Lin, and S.A. Waldman, *Central and peripheral molecular targets for antiobesity pharmacotherapy.* Clin Pharmacol Ther, 2010. **87**(6): p. 652-62.
4. Chiesi, M., C. Huppertz, and K.G. Hofbauer, *Pharmacotherapy of obesity: targets and perspectives.* Trends Pharmacol Sci, 2001. **22**(5): p. 247-54.
5. Bray, G.A. and L.A. Tartaglia, *Medicinal strategies in the treatment of obesity.* Nature, 2000. **404**(6778): p. 672-7.
6. Rosen, E.D. and B.M. Spiegelman, *Adipocytes as regulators of energy balance and glucose homeostasis.* Nature, 2006. **444**(7121): p. 847-53.
7. Spalding, K.L., et al., *Dynamics of fat cell turnover in humans.* Nature, 2008. **453**(7196): p. 783-7.
8. Drolet, R., et al., *Hypertrophy and hyperplasia of abdominal adipose tissues in women.* Int J Obes (Lond), 2008. **32**(2): p. 283-91.
9. Harp, J.B., *New insights into inhibitors of adipogenesis.* Curr Opin Lipidol, 2004. **15**(3): p. 303-7.
10. Yun, J.W., *Possible anti-obesity therapeutics from nature--a review.* Phytochemistry, 2010. **71**(14-15): p. 1625-41.
11. Tang, Q.Q. and M.D. Lane, *Adipogenesis: from stem cell to adipocyte.* Annu Rev Biochem, 2012. **81**: p. 715-36.
12. Lefterova, M.I. and M.A. Lazar, *New developments in adipogenesis.* Trends Endocrinol Metab, 2009. **20**(3): p. 107-14.
13. Siersbaek, R., R. Nielsen, and S. Mandrup, *Transcriptional networks and chromatin remodeling controlling adipogenesis.* Trends Endocrinol Metab, 2012. **23**(2): p. 56-64.
14. Tang, Q.Q., T.C. Otto, and M.D. Lane, *Mitotic clonal expansion: a synchronous process required for adipogenesis.* Proc Natl Acad Sci U S A, 2003. **100**(1): p. 44-9.

15. Tang, Q.Q., T.C. Otto, and M.D. Lane, *CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis.* Proc Natl Acad Sci U S A, 2003. **100**(3): p. 850-5.
16. Yu, W., et al., *Critical role of phosphoinositide 3-kinase cascade in adipogenesis of human mesenchymal stem cells.* Mol Cell Biochem, 2008. **310**(1-2): p. 11-8.
17. Prusty, D., et al., *Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARgamma) and C/EBPalpha gene expression during the differentiation of 3T3-L1 preadipocytes.* J Biol Chem, 2002. **277**(48): p. 46226-32.
18. Xu, J. and K. Liao, *Protein kinase B/AKT 1 plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation.* J Biol Chem, 2004. **279**(34): p. 35914-22.
19. Rosen, E.D., et al., *PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro.* Mol Cell, 1999. **4**(4): p. 611-7.
20. Wu, Z., et al., *Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity.* Mol Cell, 1999. **3**(2): p. 151-8.
21. Yeh, W.C., et al., *Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins.* Genes Dev, 1995. **9**(2): p. 168-81.
22. Dinkova-Kostova, A.T. and R.V. Kostov, *Glucosinolates and isothiocyanates in health and disease.* Trends Mol Med, 2012. **18**(6): p. 337-47.
23. Singh, S.V. and K. Singh, *Cancer chemoprevention with dietary isothiocyanates mature for clinical translational research.* Carcinogenesis, 2012. **33**(10): p. 1833-42.
24. Verkerk, R., et al., *Glucosinolates in Brassica vegetables: the influence of the food supply chain on intake, bioavailability and human health.* Mol Nutr Food Res, 2009. **53 Suppl 2**: p. S219.
25. Jadhav, U., et al., *Iberin induces cell cycle arrest and apoptosis in human neuroblastoma cells.* Int J Mol Med, 2007. **19**(3): p. 353-61.

26. Jadhav, U., et al., *Dietary isothiocyanate iberin inhibits growth and induces apoptosis in human glioblastoma cells.* J Pharmacol Sci, 2007. **103**(2): p. 247-51.
27. Jakubikova, J., et al., *Isothiocyanate iberin modulates phase II enzymes, posttranslational modification of histones and inhibits growth of Caco-2 cells by inducing apoptosis.* Neoplasma, 2006. **53**(6): p. 463-70.
28. Jakobsen, T.H., et al., *Food as a source for quorum sensing inhibitors: iberin from horseradish revealed as a quorum sensing inhibitor of Pseudomonas aeruginosa.* Appl Environ Microbiol, 2012. **78**(7): p. 2410-21.
29. Cho, E.S., et al., *Cocoa procyanidins attenuate 4-hydroxynonenal-induced apoptosis of PC12 cells by directly inhibiting mitogen-activated protein kinase kinase 4 activity.* Free Radic Biol Med, 2009. **46**(10): p. 1319-27.
30. Lane, M.D. and Q.Q. Tang, *From multipotent stem cell to adipocyte.* Birth Defects Res A Clin Mol Teratol, 2005. **73**(7): p. 476-7.
31. Resnitzky, D., et al., *Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system.* Mol Cell Biol, 1994. **14**(3): p. 1669-79.
32. Strausfeld, U.P., et al., *Both cyclin A and cyclin E have S-phase promoting (SPF) activity in Xenopus egg extracts.* J Cell Sci, 1996. **109 (Pt 6)**: p. 1555-63.
33. Mendoza, M.C., E.E. Er, and J. Blenis, *The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation.* Trends Biochem Sci, 2011. **36**(6): p. 320-8.
34. Inoue, G., et al., *Dynamics of insulin signaling in 3T3-L1 adipocytes. Differential compartmentalization and trafficking of insulin receptor substrate (IRS)-1 and IRS-2.* J Biol Chem, 1998. **273**(19): p. 11548-55.
35. Alessi, D.R., et al., *Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha.* Curr Biol, 1997. **7**(4): p. 261-9.
36. Song, M.S., L. Salmena, and P.P. Pandolfi, *The functions and regulation of the PTEN tumour suppressor.* Nat Rev Mol Cell Biol, 2012. **13**(5): p. 283-

96.

37. Yap, T.A., et al., *Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises.* Curr Opin Pharmacol, 2008. **8**(4): p. 393-412.
38. Kersten, S., *Mechanisms of nutritional and hormonal regulation of lipogenesis.* EMBO Rep, 2001. **2**(4): p. 282-6.
39. Schadinger, S.E., et al., *PPARgamma2 regulates lipogenesis and lipid accumulation in steatotic hepatocytes.* Am J Physiol Endocrinol Metab, 2005. **288**(6): p. E1195-205.
40. Porstmann, T., et al., *SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth.* Cell Metab, 2008. **8**(3): p. 224-36.
41. Fajas, L., et al., *Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: implications for adipocyte differentiation and metabolism.* Mol Cell Biol, 1999. **19**(8): p. 5495-503.
42. Lodhi, I.J., et al., *Inhibiting adipose tissue lipogenesis reprograms thermogenesis and PPARgamma activation to decrease diet-induced obesity.* Cell Metab, 2012. **16**(2): p. 189-201.
43. Ahmadian, M., R.E. Duncan, and H.S. Sul, *The skinny on fat: lipolysis and fatty acid utilization in adipocytes.* Trends Endocrinol Metab, 2009. **20**(9): p. 424-8.
44. Whittle, A., J. Relat-Pardo, and A. Vidal-Puig, *Pharmacological strategies for targeting BAT thermogenesis.* Trends Pharmacol Sci, 2013.
45. Choi, K.M., et al., *Sulforaphane inhibits mitotic clonal expansion during adipogenesis through cell cycle arrest.* Obesity (Silver Spring), 2012. **20**(7): p. 1365-71.
46. Ortega-Molina, A., et al., *Pten positively regulates brown adipose function, energy expenditure, and longevity.* Cell Metab, 2012. **15**(3): p. 382-94.
47. Chakraborty, A., et al., *Inositol pyrophosphates inhibit Akt signaling, thereby regulating insulin sensitivity and weight gain.* Cell, 2010. **143**(6): p. 897-910.
48. Dann, S.G., A. Selvaraj, and G. Thomas, *mTOR Complex1-S6K1 signaling:*

at the crossroads of obesity, diabetes and cancer. Trends Mol Med, 2007. **13**(6): p. 252-9.

49. Moore, T., et al., *Dietary energy balance modulates signaling through the Akt/mammalian target of rapamycin pathways in multiple epithelial tissues.* Cancer Prev Res (Phila), 2008. **1**(1): p. 65-76.

Ⅴ. 국문초록

이베린은 양배추, 콜리플라워, 브로콜리와 같은 십자화과 식물에서 유래하는 천연 물질로 선행 연구에서 항암 작용이 보고되었다. 하지만 이베린의 항비만효과와 그에 관한 분자 기작은 아직까지 연구된 바가 없다. 본 연구에서는 이베린이 3T3-L1 지방전구세포에서 지방 분화를 억제하는 효능을 확인하였으면 이에 관련하여 분자기전들을 규명하였다. 이베린이 분화 유도제에 의한 지방전구세포의 분화와 지방 축적을 효과적으로 억제함을 Oil Red O 염색법을 통해 확인하였다. 또한 지방 분화에 중요한 조절 단백질인 PPAR와 C/EBP의 발현이 저해됨을 확인하였다. 더 나아가, 지방분화 단계 중 초기 단계에 이베린이 가장 중요하게 작용하는 것을 확인하였고, 분화 초기 단계에서 일어나는 유사분열성세포증식을 확인하기 위해 FACS 분석을 이용하여 세포주기의 진행이 억제됨을 확인하였다. 또한 이베린이 세포증식을 조절하는 Akt 신호전달과정을 효과적으로 저해하였음을 발견하였다. 종합적으로, 본 연구에서는 이베린이 지방세포분화와 지방축적을 효과적으로 저해할 수 있는 천연 물질이며, 이는 주로 Akt 신호전달과정을 억제하여 이로 인해 저해된 유사분열성세포증식이 결국에 지방세포분화

억제로 기인한 효과임을 밝혔다.

주요어: 이베린; Akt 신호전달; 지방세포분화; 3T3-L1 지방전구세포

학 번: 2011-24115