A Thesis for the Degree of Master of Science

5-(3', 4'-Dihydroxyphenyl)-γ-valerolactone, a Metabolite of Procyanidins in Cacao, Suppresses MDI-induced Adipogenesis by Regulating Cell Cycle Progression

카카오 프로시아니딘 대사체의 세포 주기 조절을 통한 지방세포 분화 억제 효능

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ABSTRACT

Cacao (*Theobroma cacao*) which includes a large amount of polyphenols, has been reported to have potent beneficial effects on obesity. However, all of these previous studies have focused on the extract form of cacao or procyanidins and their potential mechanisms have not been fully elucidated with considering ADME (absorption, distribution, metabolism, and excretion). Here, in this study, I investigated the inhibitory effects of metabolites of procyanidins on adipogenic cocktail (MDI)-induced adipogenesis and lipogenesis in 3T3-L1 preadipocytes. Especially, 5-(3', 4'-Dihydroxyphenyl)-γ-valerolactone (DHPV) which is known for the major metabolite of proycanidins, had the best inhibitory effects on adipogenesis and lipogenesis. I found that DHPV decreased the protein expression levels which are involved in adipogenesis such as peroxisome proliferator-

activated receptor γ (PPAR γ) and CCAT/enhancer-binding protein α (C/EBP α), and lipogenesis such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) dose-dependently. These inhibitory effects were mainly due to G1 phase arrest in the early stage of adipogenesis which called as mitotic clonal expansion (MCE) by suppressing cell proliferation. In kinase array, CDK2/cyclin O was preferentially selected through several citeria. DHPV directly suppressed activation of CDK2/cyclin O complex. DHPV inhibited the phosphorylation of C/EBP β which is responsible for induction of PPAR γ and C/EBP α. Taken together, this study implied that DHPV is one of the biologically active compound of cacao which brings the potential benefits on anti-obesity by inhibiting intracellular lipid contents and cell differentiation.

Keywords: Obesity; Adipogenesis; Metabolite; CDK2/cyclin O; 5-(3', 4'-Dihydroxyphenyl)-γ-valerolactone; DHPV;

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CONTENTS

Ⅰ. INTRODUCTION

Obesity is one of the biggest health problems worldwide, because it can lead a risk of severe human diseases such as cancer, hypertension, and type 2 diabetes [1, 2]. As its prevalence is constantly growing, numerous strategies and drugs are appeared to prevent or treat obesity [3-5]. However, it has been reported that there are many side effects of the anti-obesity drugs. Therefore, it is important to find the compounds which have both very few side effects and much greater effects.

Recent studies have focused on the anti-obesity effects of natural compounds such as polyphenols [6, 7]. Cacao (*Theobroma cacao*) which is the essential and main ingredient of the chocolate, is also known for including a large amount of polyphenols [8]. Interestingly, cacao has more phenolic and flavonoid contents than teas

and red wine [9]. Among these cacao polyphenols, the procyanidins are reported to be the most abundant types of polyphenol group. Due to this high concentration of procyanidins, cacao could provide the preventative effects [10]. It has been reported that cacao could contribute the various potential health benefits such as cardiovascular disease [11], cancer [12], neuronal disease [13], and diabetes [14]. Furthermore, the anti-obesity effects of cacao have steadily been reported [15-18]. However, all of these previous researches are limited to studying the extract form of cacao or procyanidins.

Numerous studies are revealed the anti-obesity effects of cacao which contains high content of total procyanidins [17, 19]. However, the many functions of biologically activities may not result in the systemic bioavailability of procyanidins. It has been suggested that it may have minimal effects in vivo study due to intestinal decomposition [20]. Therefore, it is essentially considered in terms of absorption,

distribution, metabolism, and excretion (ADME). That is, metabolites of procyanidins might play a very key role in the overall effects of native compounds of procyanidins [10]. Additionally, it is important that which concentration of metabolites remains in blood and plasma for an extended period of time.

In the present study, I focused on metabolites of procyanidins which bring the potential benefits on anti-obesity. I investigated the beneficial effects of procyanidins and their metabolites. According to previous studies, procyanidins are metabolized to (+)-Catechin, (-)-Epicatechin, 2-(3, 4-Dihydroxyphenyl)-acetic acid (DHPAA), 2-(3-Hydroxyphenyl)-acetic acid (HPAA), 3-(3, 4-Dihydroxyphenyl)-propionic acid (DHHC), 3-(3-Hydroxyphenyl)-propionic acid (HC), and 5-(3', 4'-Dihydroxyphenyl)-γ-valerolactone (DHPV) as metabolites [10, 21-23].

First, I examined the anti-adipogenic effects of procyanidins

and their metabolites on MDI-induced adipogenesis. Of the 9 compounds, DHPV most strongly caused the inhibitory effect on adipogenesis in 3T3-L1 preadipocytes. Interestingly, DHPV is been reported that exists a high concentration in fasting plasma [21]. DHPV was found to block cell cycle progression, thereby inhibiting mitotic clonal expansion. DHPV also inhibited CDK2/cyclin O activity and suppressed adipogenesis and lipogenesis-related biomarkers. These results demonstrated that DHPV attenuated adipogenesis and lipogenesis in 3T3-L1 preadipocytes.

Ⅱ. MATERIALS AND METHODS

2.1. Chemicals and reagents

Procyanidin B2 was purchased from Funakoshi Co. (Funakoshi, Japan). Procyanidin B3, (+)-Catechin, (-)-Epicatechin, 2-(3, 4-Dihydroxyphenyl)-acetic acid, 2-(3-Hydroxyphenyl)-acetic acid, 3-(3, 4-Dihydroxyphenyl)-propionic acid, isobutylmethylxanthine (IBMX), dexamethasone, and human insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(3-Hydroxyphenyl)-propionic acid was purchased from Alfa aeser Co. (Incheon, South Korea). 5-(3', 4'-Dihydroxyphenyl)-γ-valerolactone was purchased from Chemieliva Co. (Chondqing, China). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and bovine calf serum (BCS) were purchased from GIBCO (Grand Island, NY).

Antibody against peroxisome proliferator-activated receptor γ

(PPAR γ), cyclin-dependent kinase2 (CDK2), cyclin O, β-actin, and CCAAT/enhancer-binding proteins α and β (C/EBP α and β) were purchased from Santa cruz biotechnology (Santacruz, CA).

Fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), sterol regulatory element-binding protein 1c (SREBP1c), and phospho-CCAAT/enhancer-binding proteins β (p-C/EBP β) were purchased from Cell signaling Technology (Danvers, MA, USA).

2.2. Cell culture and preadipocytes differentiation

3T3-L1 preadipocytes were purchased from American Type Culture Collection (ATCC). 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% BCS, a 5% CO_2, and 37 ℃ until 100% confluence. After post-confluent (day 0), cells were incubated in DMEM supplemented with 10% fetal bovine serum (FBS) and adipogenic cocktail (MDI) which was a mixture of 0.5 mM 3-isobutyl-

1-methylxanthine (IBMX), 1 μM dexamethasone (DEX), and 5 μg/mL insulin for 2 days in order to induce differentiation. After 2 days, medium was changed to DMEM containing 10% FBS and 5 μg/mL insulin. 2 days later, medium were switched to DMEM containing 10% FBS until preadipocytes were fully differentiated.

2.3. Cell viability assay

In order to assess cell viability, 3T3-L1 preadipocytes were seeded in 24 well plate at a density of 5 x 10^4 cells per well. After confluence, cells were treated with 10% FBS DMEM supplemented with DHPV or only 10% FBS DMEM as a control. A 10% of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] solution (Cleveland, OH, USA) of total medium was added to each well, and the cells were incubated for 1 hour. The medium was removed and the dark formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The

absorbance at 570 nm was measured with spectrophotometry. The results were expressed as percent MTT reduction relative to the absorbance of the control cells.

2.4. Oil Red O staining

In order to evaluate accumulated lipid contents in the cells, 3T3-L1 preadipocytes were seeded in 24 well plate at a density of 5 x 10^4 cells per well. After confluence, the cells were differentiated for 6 days with or without compounds. Media were removed and differentiated cells were fixed with 4% formalin for 20 min and washed with the phosphate buffered saline (PBS). The fixed cells were then stained with Oil Red O solution for 15 min at room temperature in the dark. Oil Red O solution was prepared by dissolving 0.25 mg Oil Red O powder (Sigma-Aldrich, St. Louis, MO, USA) in 50 mL 60% isopropyl alcohol (Amresco, Solon, OH) followed by filtering with

0.45 μM membrane. After staining, the cells were washed 3 times with PBS. Intracellular lipid contents were quantified by eluting Oil Red O staining with isopropyl alcohol and measured at 515 nm with spectrophotometry. The results were expressed as percent Oil Red O staining reduction relative to the absorbance of the control cells.

2.5. Glycerol assay

In order to evaluate glycerol releases, 3T3-L1 preadipocytes were differentiated in 24 well plates as described above. The cells were then treated with 10% FBS DMEM supplemented with or without 5, 10, and 20 μM DHPV and isoproterenol (Sigma-Aldrich, St. Louis, MO, USA) as a positive control [24] for 3 days. Next, the cell-free culture supernatants were transferred to clear 96 well plates. Glycerol concentrations were measured using Free Glycerol Reagent (Sigma-Aldrich, St. Louis, MO, USA) as described by the manufacturer.

2.6. Western blot analysis

After 3T3-L1 preadipocytes were cultured in 6 cm dishes at a density of 5 x 10^4 cells per dish for 2 days in DMEM supplemented with 10% BCS. When cells reached confluence, medium was changed with DMEM supplemented with 10% FBS and MDI hormone cocktail containing with or without 5, 10, and 20 μM DHPV. The cells were washed with PBS and harvested by scraping in cell lysis buffer. The protein concentration was measured using a protein assay reagent kit (Hercules, CA) as described by the manufacturer. The proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (GE healthcare, Piscataway, NJ). The membranes were blocked in 5% skim milk for 1 hour, and then incubated with a specific primary antibody at 4 ℃ overnight. Protein bands were visualized by a chemiluminescence detection kit (GE Healthcare, London, UK) after

hybridization with a HRP-conjugated secondary antibody (Life Technologies, Waltham, MA).

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

In order to analyze cell cycle, 3T3-L1 preadipocytes were seeded in 6 cm dishes a density of 5 x 10^4 cells per dish and cultured until confluence. And then, cells were incubated in DMEM supplemented with 10% FBS, 0.5 mM IBMX, 1 μM DEX, 5 μg/mL insulin and with and without 20 μM DHPV for 0, 8, 16, 20, 24, and 48 hour, respectively. After, cells were trypsinized and centrifuged at 1000 rpm for 3 min. The pellets were re-suspended in PBS and centrifuged at 1000 rpm for 3 min again. The pellets were suspended in cold 70% (v/v) ethanol (Sigma-Aldrich, St. Louis, MO, USA) for fixing and maintained at 4 ℃ overnight. And then, cells were centrifuged at 1500

rpm for 3 min and re-suspended in 700 μL of PBS containing 10 μg/mL PI solution (Sigma-Aldrich, St. Louis, MO, USA) and 0.2 mg/ml RNase (Amresco, Solon, OH). Cells were incubated at 37 ℃ for 15 min in the dark. Finally, fluorescence emitted from cells was measured using a flow cytometer (FACSCalibur, Becton-Kickinson, San Jose, CA, USA) with CellQuest software (BC Biosciences). Ten thousand cells in each sampled were analyzed [25].

2.8. Kinase assay

In order to find the molecular targets of DHPV, kinase profiling were conducted by kinase assay services (Reaction Biology Corporation). Briefly, kinase was incubated with substrate and required cofactors. The reaction was initiated by the addition of the compound in DMSO and ^{33}P-ATP (specific activity 10 μCi/μl). After incubation for 120 min at room temperature, the reaction was spotted onto P81 ion

exchange paper (GE Healthcare) and washed extensively in 0.75% phosphoric acid. Kinase activity data were expressed as the percent remaining kinase activity in test samples compared to vehicle (DMSO) reactions.

2.9. Pull-down assay

Sepharose 4B freeze-dried powder was purchased from GE Healthcare (Little Chalfont, UK). Sepharose 4B beads (0.3 g) were activated in 1 mM hydrogen chloride (HCl) and suspended in DHPV (2 mg) coupled solution (0.1 M $NaHCO_3$ and 0.5 M NaCl). Following overnight rotation at 4 ℃, the mixture was transferred to 0.1 M Tris-HCl buffer (pH 8.0) and rotated at 4 ℃ overnight again. The mixture was then washed with 0.1 M acetated buffer (pH 4.0), 0.1 M Tris-HCl, 0.5 M NaCl buffer (pH 8.0) and suspended in PBS. After beads were ready for use, CDK2/cyclin O active protein (Signalchem, Richmond,

Canada) was incubated with either Sepharose 4B alone or DHPV-Sepharose 4B beads in reaction buffer. After incubation at 4 ℃, the beads were washed with washing buffer and the proteins bound to the beads were analyzed by western blot.

2.10. Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Duncan's Multiple Range Test, and p values of less than 0.05 were considered statistically significant.

Ⅲ. RESULTS

3.1. DHPV had the most potent inhibitory effects on MDI-induced adipogenesis in 3T3-L1 preadipocytes.

The structure and tentative pathway for human microbial degradation of procyanidins in cacao showed in Figure 1A [34, 35]. To investigate the anti-adipogenic effects of procyanidins and their metabolites, 3T3-L1 preadipocytes were incubated with each procyanidins and their metabolites simultaneously with 20 μM for 6 days. Lipid contents were quantified with Oil Red O staining. MDI-treated differentiated cells were significantly increased the relative lipid contents by 6-fold compared to undifferentiated cells as control. ORO staining result showed that DHPV most strongly reduced lipid contents among other procyanidins and their metabolites (Fig. 1B). To determine whether the decreased lipid contents was a result of diminishing cell

number, I examined the cell viability of each procyanidins and their metabolites using MTT assay. As a result, none of compounds did not decline cell viability (Fig. 1C).

Figure 1

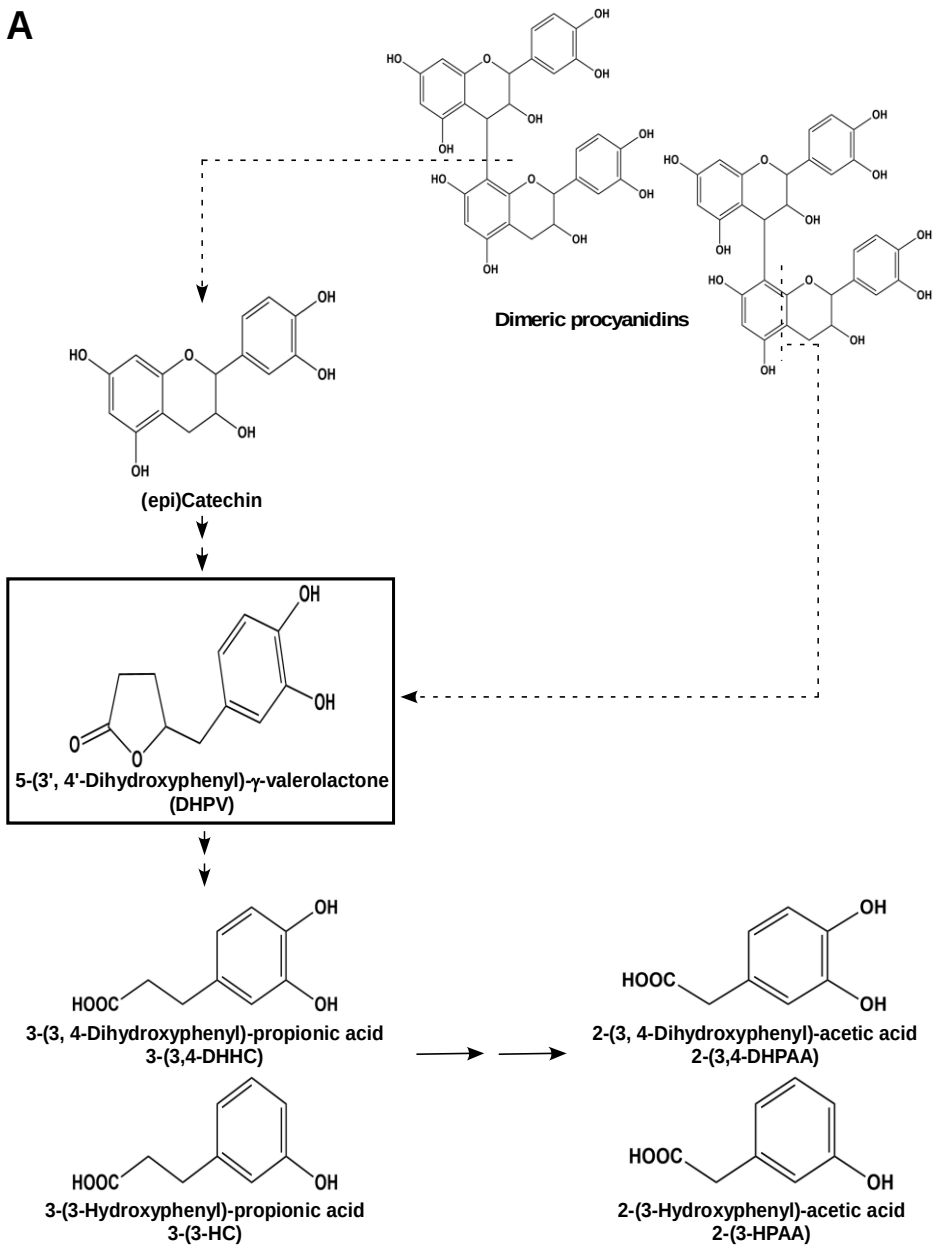
A
Dimeric procyanidins
(epi)Catechin
5-(3', 4'-Dihydroxyphenyl)-γ-valerolactone
(DHPV)
3-(3, 4-Dihydroxyphenyl)-propionic acid
3-(3,4-DHHC)
2-(3, 4-Dihydroxyphenyl)-acetic acid
2-(3,4-DHPAA)
3-(3-Hydroxyphenyl)-propionic acid
3-(3-HC)
2-(3-Hydroxyphenyl)-acetic acid
2-(3-HPAA)

Figure 1. continue

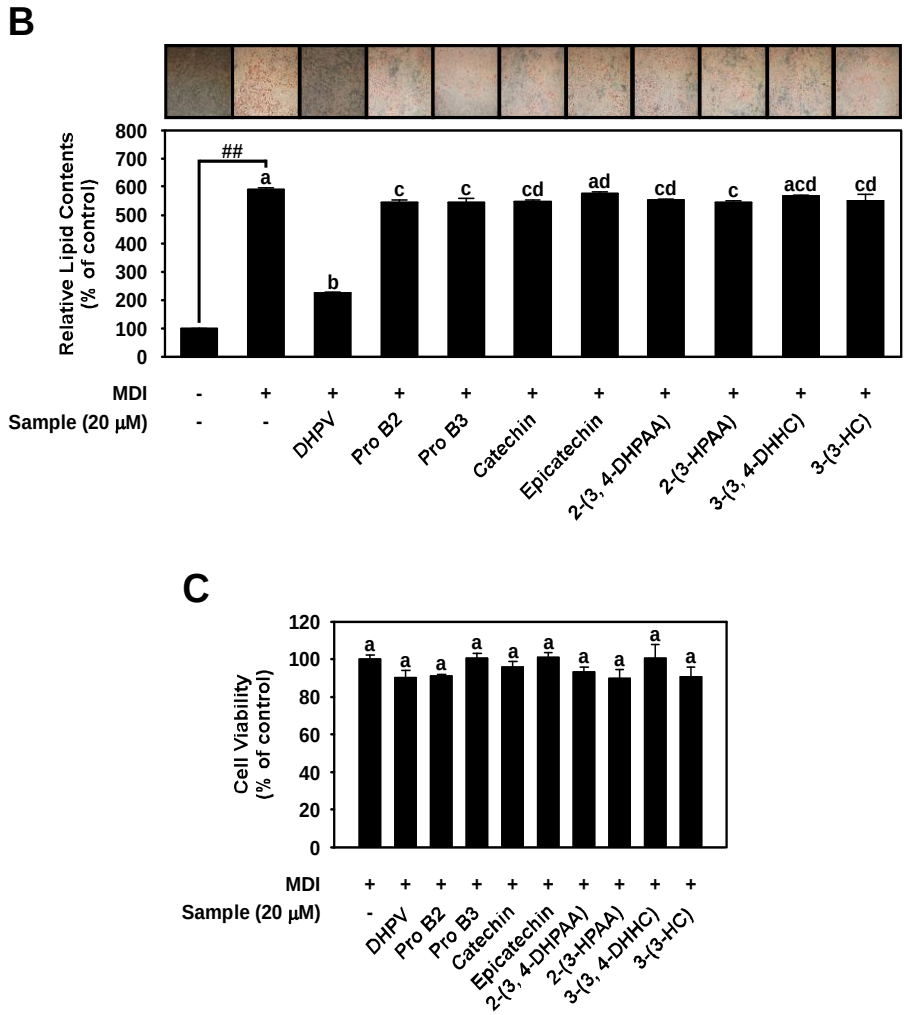

Figure 1. The greatest inhibitory effects of DHPV on MDI-induced adipogenesis in 3T3-L1 preadipocytes

(A) Provisional degradation pathway for procyanidins (B) Oil Red O staining images and data showed that DHPV significantly inhibited intracellular lipid accumulation in 3T3-L1 preadipocytes among other procyanidins such as procyanidins B2 and B3 which are known for rich in cacao [26, 27], and their metabolites such as (+)-Catechin, (-)-Epicatechin, 2-(3, 4-DHPAA), 2-(3-HPAA), 3-(3, 4-DHHC), 3-(3-HC), and DHPV. (C) All above compounds did not affect on cell viability. Data were represented as mean ± standard error of mean (SEM) values of at least three independent experiments. The sharps (##) indicated a significant difference between the control group and the group treated with MDI cocktail alone ($p < 0.01$). To compare the differences among the groups, one-way ANOVA was used with Duncan's multiple range tests. Different letters indicated statistically

significant differences at $p < 0.05$.

3.2. DHPV inhibited the adipogenic and lipogenic effects in 3T3-L1 preadipocytes.

To investigate the effects of DHPV on adipogenesis in 3T3-L1 preadipocytes, cells were treated with or without DHPV. Differentiation of 3T3-L1 preadipocytes were induced by the method as previously described and lipid contents were quantified with Oil Red O staining. As a result, compared to undifferentiated cells, the relative lipid contents of MDI-induced cells were increased by 8-fold. DHPV treated group reduced intracellular lipid contents dose-dependently at 5, 10, and 20 μM to about 41%, 63%, and 72%, respectively (Fig. 2A).

Consistent with this result, I also examined the MDI-induced expression of proteins, which are marker proteins of adipogenesis [28] and lipogenesis [29]. Western blot data showed that DHPV suppressed the protein expression levels of PPAR γ and C/EBP α which are key regulators of adipogenesis (Fig. 2B). Furthermore, DHPV suppressed

the expression levels of SREBP1c, ACC, and FAS which are lipogenic proteins (Fig. 2C). Next, I performed MTT assay to investigate the toxic effect of DHPV on cell viability in 3T3-L1 preadipocytes. As a result, DHPV did not have toxicity up to 20 μM (Fig. 2D).

Figure 2

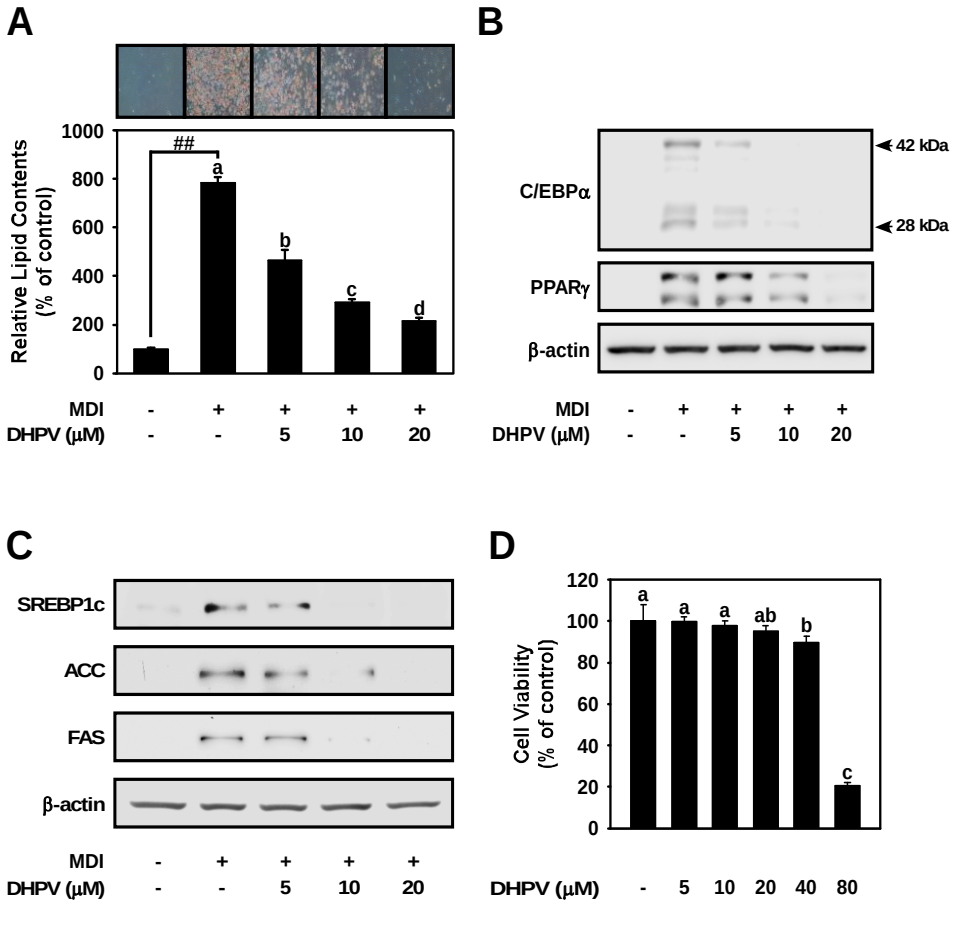
A
Relative Lipid Contents (% of control)
1000
800
600
400
200
0
##
a
b
c
d
MDI - + + + +
DHPV (μM) - - 5 10 20
B
C/EBPα
PPARγ
β-actin
42 kDa
28 kDa
MDI - + + + +
DHPV (μM) - - 5 10 20
C
SREBP1c
ACC
FAS
β-actin
MDI - + + + +
DHPV (μM) - - 5 10 20
D
Cell Viability (% of control)
120
100
80
60
40
20
0
a
a
a
ab
b
c
DHPV (μM) - 5 10 20 40 80

Figure 2. Effects of DHPV on MDI-induced adipogenesis and lipogenesis 3T3-L1 preadipocytes

(A) Oil Red O staining images and data showed that DHPV significantly inhibited intracellular lipid accumulation in 3T3-L1 preadipocytes. (B) DHPV down-regulated the protein expression levels of adipogenesis such as PPAR γ and C/EBP α. (C) DHPV down-regulated the protein expression levels of lipogenesis such as SREBP1c, ACC and FAS. (D) DHPV did not affect on cell viability up to 20 μM. Data were represented as mean ± standard error of mean (SEM) values of at least three independent experiments. The sharps (##) indicated a significant difference between the control group and the group treated with MDI cocktail alone ($p < 0.01$). To compare the differences among the groups, one-way ANOVA was used with Duncan's multiple range tests. Different letters indicated statistically significant differences at $p < 0.05$.

3.3. DHPV inhibited MDI-induced adipogenesis at mitotic clonal expansion step in 3T3-L1 preadipocytes.

After growth arrested by contact inhibition, preadipocytes progress to cell differentiation called the adipogenesis. This process is divided into two broad stages, the mitotic clonal expansion which increases cell number, and the terminal differentiation which increases intracellular lipid contents, respectively [30, 31]. Therefore, I investigated which the critical time-point of the inhibitory effect of DHPV on adipocyte differentiation. To elucidate which stage in adipogenesis is affected by DHPV, I treated DHPV 20 μM at different periods of adipogenesis during 6 days for full differentiation (Fig. 3A). As expected, cells treated with DHPV for 6 days showed a complete inhibition of lipid contents like previous study (Fig. 2A). All stages were moderately inhibited of lipid contents, however interestingly, DHPV suppressed adipogenesis effectively when it was treated on the

duration including 0-2 days (Fig. 3B). This result indicated that DHPV primarily modulated in the early stage of differentiation, in which mitotic clonal expansion occurred.

Figure 3

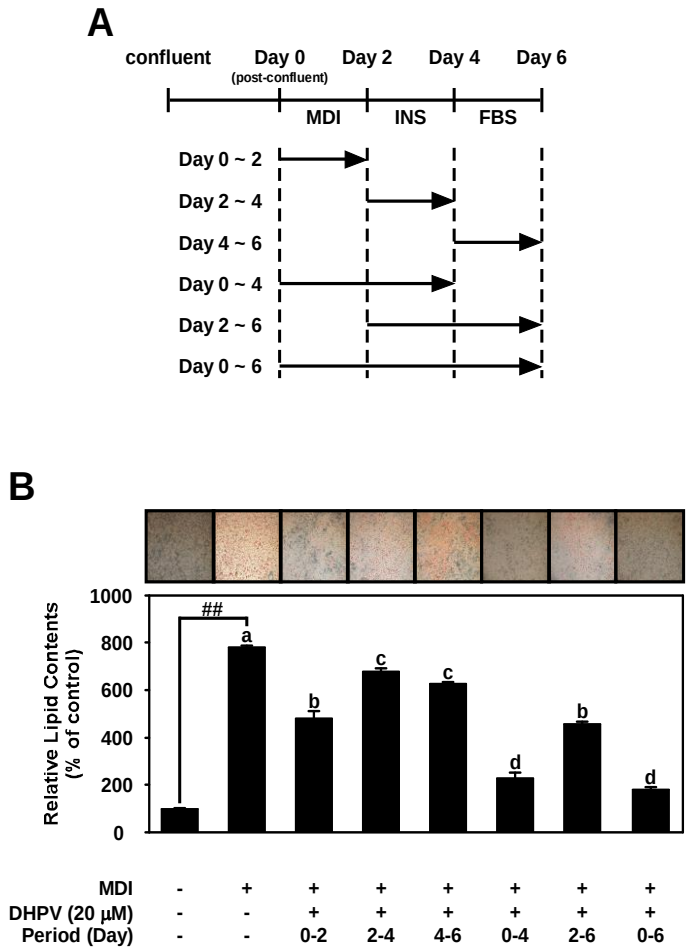
A
confluent
Day 0
(post-confluent)
Day 2
Day 4
Day 6
MDI
INS
FBS
Day 0 ~ 2
Day 2 ~ 4
Day 4 ~ 6
Day 0 ~ 4
Day 2 ~ 6
Day 0 ~ 6
B
Relative Lipid Contents
(% of control)
1000
800
600
400
200
0
##
a
b
c
c
d
b
d
MDI - + + + + + + +
DHPV (20 μM) - - + + + + + +
Period (Day) - - 0-2 2-4 4-6 0-4 2-6 0-6

Figure 3. Effects of DHPV on MDI-induced mitotic clonal expansion in the early stage of adipogenesis

(A) A schematic diagram of experiment; time schedule for periods treated DHPV for each group (B) Oil Red O staining images and data showed that DHPV had the strong anti-adipogenic effect when it was treated period including 0-2 days. Data were represented as mean ± standard error of mean (SEM) values of at least three independent experiments. The sharps (##) indicated a significant difference between the control group and the group treated with MDI cocktail alone ($p < 0.01$). To compare the differences among the groups, one-way ANOVA was used with Duncan's multiple range tests. Different letters indicated statistically significant differences at $p < 0.05$.

3.4. DHPV blocked the cell cycle progression by inducing G1 arrest in 3T3-L1 preadipocytes.

Continuously I investigated how DHPV inhibited the mitotic clonal expansion, which is known as a prerequisite stage in early adipogenesis [32]. To confirm that DHPV directly made impact on proliferation, I performed FACS analysis to investigate cell cycle progression on 3T3-L1 preadipocytes during mitotic clonal expansion. According to FACS analysis data, MDI-induced cell cycle progression was blocked by DHPV compared to the differentiated groups (Fig. 4A). At 16 hr, the MDI-treated cells moved S phase compared to control. Then, distinctively the number of cells in S phase were increased by MDI. However, the cells treated MDI with DHPV did not undergo cell cycle progression and stayed in basal stage, G1 phase, which was the same as control groups. (Fig. 4B). These results indicated that DHPV delayed cell cycle progression from G1 phase to S phase in the early

stage of differentiation.

Figure 4

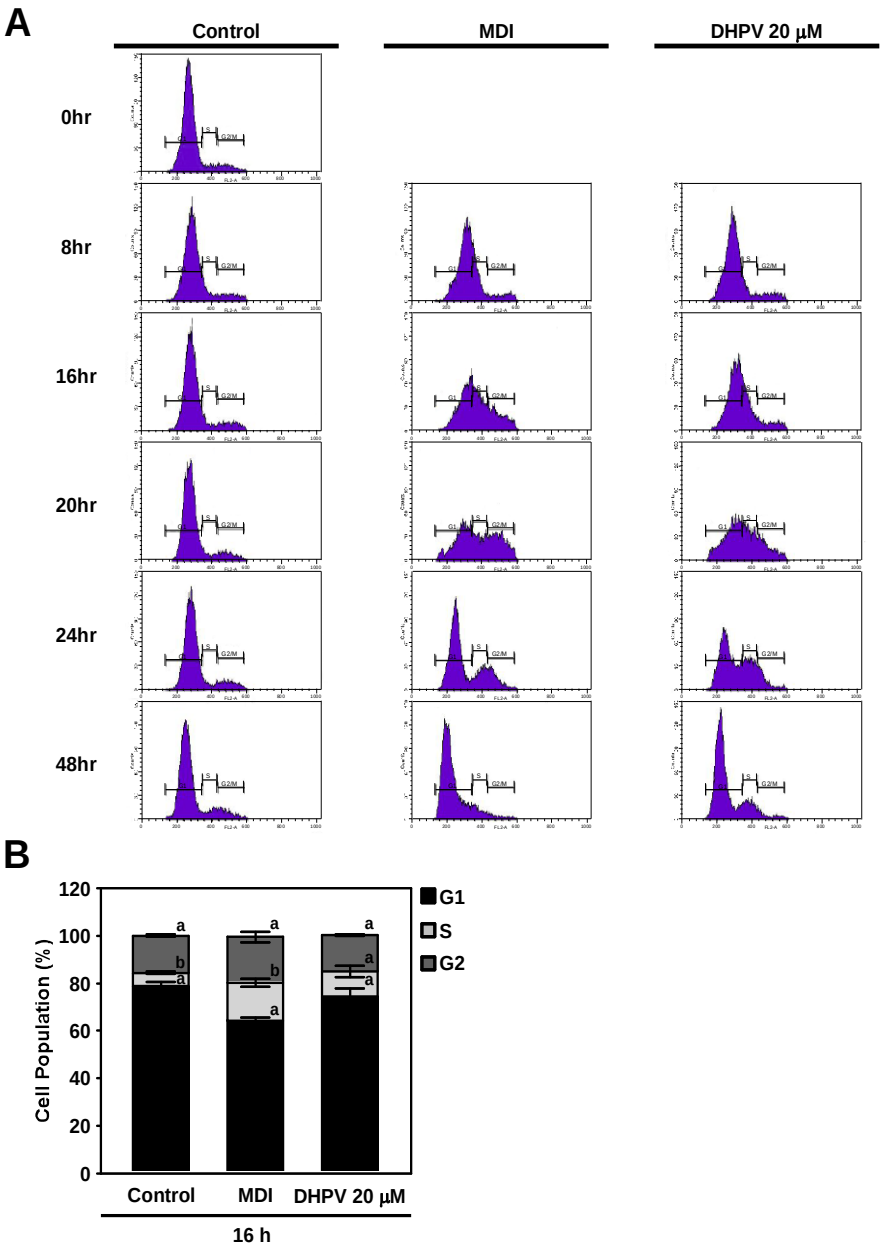
A
Control
MDI
DHPV 20 μM
0hr
8hr
16hr
20hr
24hr
48hr
B
Cell Population (%)
120
100
80
60
40
20
0
G1
S
G2
Control
MDI
DHPV 20 μM
16 h

Figure 4. Effects of DHPV blocked the G1 phase during mitotic clonal expansion.

(A) FACS analysis histograms and (B) quantitative data showed that DHPV blocked the G1 phase in the early stage of differentiation. Data were represented as mean ± standard error of mean (SEM) values of at least three independent experiments. To compare the differences among the groups, one-way ANOVA was used with Duncan's multiple range tests. Different letters indicated statistically significant differences at $p < 0.05$.

3.5. DHPV directly inhibited CDK2/cyclin O activity.

To elucidate the molecular mechanisms about how DHPV had the inhibitory effects, I performed kinase profiling analysis. First, I tested in duplicate mode at a concentration of DHPV 10 μM (Table 1). Based on Table 1, 6 kinases were selected, which were inhibited more than 65% by DHPV. Next, I tested in a 10-dose IC_{50} mode with 3-fold serial dilution starting at DHPV 160 μM (Table 2). Additionally, based on the results from Table 2, I considered these following requirements. Target kinases should regulate to cell cycle, were expressed in mouse and adipocytes, and were related to MDI-induced signaling. As a result, I preferentially chosen CDK2/cyclin O kinase for potential target of DHPV.

DHPV effectively inhibited CDK2/cyclin O activity with the half maximal inhibitory concentration (IC_{50}) (IC_{50} = 8.79 μM) (Fig. 5A). To demonstrate whether DHPV directly interacted with CDK2/cyclin O

then inhibited its activity, I performed pull-down assay using CDK2/cyclin O active protein. As a result, DHPV could directly bind to CDK2/cyclin O (Fig. 5B).

Continuously I investigated how CDK2/cyclin O affected during MDI-induced adipogenesis. According to previous study, CDK2 acts sequentially phosphorylation and activation of C/EBP β during adipocyte differentiation [33]. C/EBP β protein plays an important role in mitotic clonal expansion [34, 35] and also a key transcription factor for transcriptional activation of C/EBP α and PPAR γ [34-37], which are master regulators of adipogenesis and expression in terminal differentiation. Therefore, I examined the protein expression of C/EBP β. As a result, DHPV reduced phosphorylation of C/EBP β (Fig. 5C). These results implied DHPV inhibited mitotic clonal expansion by suppressing C/EBP β which is not only a downstream kinase of CDK2/cyclin O but also a transcription factor of C/EBP α and PPAR γ.

Table 1. Kinase profiling of DHPV (10 μM)

Kinase profiling analysis was performed on whole human type kinases (359 kinases) to find the target of DHPV (10 μM) by kinase assay service (Reaction Biology Corporation). Data were representative of two independent experiments that gave similar results.

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
ABL1	105	**ALK2/ACVR1**	84	**Aurora A**	79	**BRK**	103	**CAMK1a**	69
ABL2/ARG	106	**ALK3/BMPR1A**	85	**Aurora B**	93	**BRSK1**	100	**CAMK1b**	89
ACK1	89	**ALK4/ACVR1B**	75	**Aurora C**	100	**BRSK2**	89	**CAMK1d**	82
AKT1	85	**ALK5/TGFBR1**	52	**AXL**	111	**BTK**	101	**CAMK1g**	103
AKT2	84	**ALK6/BMPR1B**	79	**BLK**	105	**c-Kit**	87	**CAMK2a**	93
AKT3	118	**ARAF**	94	**BMPR2**	101	**c-MER**	77	**CAMK2b**	86
ALK	93	**ARK5/NUAK1**	102	**BMX/ETK**	91	**c-MET**	87	**CAMK2d**	101
ALK1/ACVRL1	69	**ASK1/MAP3K5**	101	**BRAF**	101	**c-Src**	106	**CAMK2g**	66

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
CAMK4	94	**CDK2/cyclin A**	81	**CDK7/cyclin H**	114	**CK1g3**	102	**DAPK2**	91
CAMKK1	115	**CDK2/Cyclin A1**	102	**CDK9/cyclin K**	95	**CK2a**	116	**DCAMKL1**	107
CAMKK2	101	**CDK2/cyclin E**	85	**CDK9/cyclin T1**	96	**CK2a2**	76	**DCAMKL2**	95
CDC7/DBF4	103	**CDK2/cyclin O**	39	**CHK1**	91	**CLK1**	95	**DDR1**	103
CDK1/cyclin A	106	**CDK3/cyclin E**	60	**CHK2**	117	**CLK2**	93	**DDR2**	100
CDK1/cyclin B	96	**CDK4/cyclin D1**	93	**CK1a1**	94	**CLK3**	91	**DLK/MAP3K12**	95
CDK1/cyclin E	100	**CDK4/cyclin D3**	92	**CK1a1L**	87	**CLK4**	102	**DMPK**	111
CDK14/cyclin Y	99	**CDK5/p25**	48	**CK1d**	83	**COT1/MAP3K8**	95	**DMPK2**	96
CDK16/cyclin Y	91	**CDK5/p35**	96	**CK1epsilon**	118	**CSK**	95	**DRAK1/STK17A**	94
CDK17/cyclin Y	102	**CDK6/cyclin D1**	93	**CK1g1**	84	**CTK/MATK**	94	**DYRK1/DYRK1A**	96
CDK18/cyclin Y	95	**CDK6/cyclin D3**	92	**CK1g2**	94	**DAPK1**	122	**DYRK1B**	101

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
DYRK2	79	**EPHA8**	97	**ERN2/IRE2**	91	**FLT4/VEGFR3**	91	**GRK6**	76
DYRK3	99	**EPHB1**	87	**FAK/PTK2**	91	**FMS**	104	**GRK7**	99
DYRK4	73	**EPHB2**	90	**FER**	88	**FRK/PTK5**	78	**GSK3a**	89
EGFR	93	**EPHB3**	96	**FES/FPS**	97	**FYN**	110	**GSK3b**	98
EPHA1	90	**EPHB4**	95	**FGFR1**	91	**GCK/MAP4K2**	103	**Haspin**	90
EPHA2	97	**ERBB2/HER2**	89	**FGFR2**	98	**GLK/MAP4K3**	92	**HCK**	96
EPHA3	104	**ERBB4/HER4**	88	**FGFR3**	95	**GRK1**	105	**HGK/MAP4K4**	95
EPHA4	94	**ERK1**	102	**FGFR4**	88	**GRK2**	100	**HIPK1**	117
EPHA5	88	**ERK2/MAPK1**	92	**FGR**	91	**GRK3**	85	**HIPK2**	94
EPHA6	101	**ERK5/MAPK7**	114	**FLT1/VEGFR1**	97	**GRK4**	87	**HIPK3**	99
EPHA7	95	**ERK7/MAPK15**	96	**FLT3**	98	**GRK5**	100	**HIPK4**	114

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
HPK1/MAP4K1	114	**JAK2**	97	**LCK**	90	**MAPKAPK3**	104	**MEKK3**	104
IGF1R	100	**JAK3**	100	**LCK2/ICK**	100	**MAPKAPK5/PRAK**	92	**MEKK6**	97
IKKa/CHUK	94	**JNK1**	87	**LIMK1**	96	**MARK1**	118	**MELK**	104
IKKb/IKBKB	103	**JNK2**	97	**LIMK2**	98	**MARK2/PAR-1Ba**	104	**MINK/MINK1**	118
IKKe/IKBKE	99	**JNK3**	95	**LKB1**	154	**MARK3**	128	**MKK4**	95
IR	96	**KDR/VEGFR2**	100	**LOK/STK10**	93	**MARK4**	110	**MKK6**	116
IRAK1	123	**KHS/MAP4K5**	95	**LRRK2**	86	**MEK1**	105	**MKK7**	108
IRAK4	105	**KSR1**	100	**LYN**	106	**MEK2**	103	**MLCK/MYLK**	100
IRR/INSRR	98	**KSR2**	98	**LYN B**	90	**MEK3**	104	**MLCK2/MYLK2**	99
ITK	99	**LATS1**	97	**MAK**	105	**MEKK1**	105	**MLK1/MAP3K9**	100
JAK1	84	**LATS2**	79	**MAPKAPK2**	129	**MEKK2**	85	**MLK2/MAP3K10**	105

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
MLK3/MAP3K11	96	**MST3/STK24**	122	**NEK5**	92	**p70S6K/RPS6KB1**	98	**PDGFRb**	94
MLK4	96	**MST4**	102	**NEK6**	107	**p70S6Kb/RPS6KB2**	93	**PDK1/PDPK1**	109
MNK1	92	**MUSK**	92	**NEK7**	121	**PAK1**	101	**PEAK1**	96
MNK2	96	**MYLK3**	98	**NEK9**	91	**PAK2**	103	**PHKg1**	115
MRCKa/CDC42BPA	102	**MYO3A**	92	**NIM1**	103	**PAK3**	105	**PHKg2**	95
MRCKb/CDC42BPB	97	**MYO3b**	97	**NLK**	113	**PAK4**	116	**PIM1**	98
MSK1/RPS6KA5	104	**NEK1**	66	**OSR1/OXSR1**	94	**PAK5**	102	**PIM2**	100
MSK2/RPS6KA4	88	**NEK11**	118	**P38a/MAPK14**	116	**PAK6**	108	**PIM3**	91
MSSK1/STK23	91	**NEK2**	85	**P38b/MAPK11**	106	**PASK**	94	**PKA**	99
MST1/STK4	90	**NEK3**	111	**P38d/MAPK13**	97	**PBK/TOPK**	107	**PKAcb**	111
MST2/STK3	106	**NEK4**	106	**P38g**	95	**PDGFRa**	98	**PKAcg**	103

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
PKCa	87	**PKCzeta**	103	**PLK4/SAK**	107	**ROS/ROS1**	101	**SLK/STK2**	107
PKCb1	103	**PKD2/PRKD2**	90	**PRKX**	100	**RSK1**	98	**SNARK/NUAK2**	129
PKCb2	82	**PKG1a**	90	**PYK2**	92	**RSK2**	94	**SRMS**	89
PKCd	131	**PKG1b**	90	**RAF1**	101	**RSK3**	96	**SRPK1**	99
PKCepsilon	92	**PKG2/PRKG2**	79	**RET**	154	**RSK4**	105	**SRPK2**	111
PKCeta	86	**PKN1/PRK1**	287	**RIPK2**	93	**SGK1**	83	**SSTK/TSSK6**	118
PKCg	84	**PKN2/PRK2**	119	**RIPK3**	98	**SGK2**	123	**STK16**	104
PKCiota	100	**PKN3/PRK3**	103	**RIPK5**	76	**SGK3/SGKL**	134	**STK21/CIT**	110
PKCmu/PRKD1	89	**PLK1**	71	**ROCK1**	101	**SIK1**	76	**STK22D/TSSK1**	112
PKCnu/PRKD3	106	**PLK2**	76	**ROCK2**	97	**SIK2**	98	**STK25/YSK1**	109
PKCtheta	107	**PLK3**	68	**RON/MST1R**	97	**SIK3**	108	**STK32B/YANK2**	107

Table 1. continue

Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity	Kinase	Activity
STK32C/YANK3	103	**TAOK3/JIK**	110	**TNK1**	93	**TYK1/LTK**	99	**WNK1**	107
STK33	97	**TBK1**	102	**TRKA**	86	**TYK2**	89	**WNK2**	96
STK38/NDR1	64	**TEC**	107	**TRKB**	97	**TYRO3/SKY**	86	**WNK3**	105
STK38L/NDR2	102	**TESK1**	94	**TRKC**	94	**ULK1**	111	**YES/YES1**	100
STK39/STLK3	96	**TGFBR2**	105	**TSSK2**	104	**ULK2**	139	**YSK4/MAP3K19**	106
SYK	101	**TIE2/TEK**	71	**TSSK3/STK22C**	100	**ULK3**	101	**ZAK/MLTK**	104
TAK1	99	**TLK1**	105	**TTBK1**	123	**VRK1**	91	**ZAP70**	100
TAOK1	98	**TLK2**	79	**TTBK2**	127	**VRK2**	62	**ZIPK/DAPK3**	96
TAOK2/TAO1	104	**TNIK**	104	**TXK**	89	**WEE1**	113		

Table 2. Kinase IC_{50} of DHPV

Based on Table 1, 6 kinases were selected because of inhibiting more than 65% kinase activity. DHPV were tested in a 10-dose IC_{50} mode with 3-fold serial dilution starting at DHPV 160 μM.

Kinase	Activity
CDK5/p25	6.04
CDK2/cyclin O	8.79
STK38/NDR1	18.4
ALK5/TGFBR1	20.8
CDK3/cyclin E	30.6

Figure 5

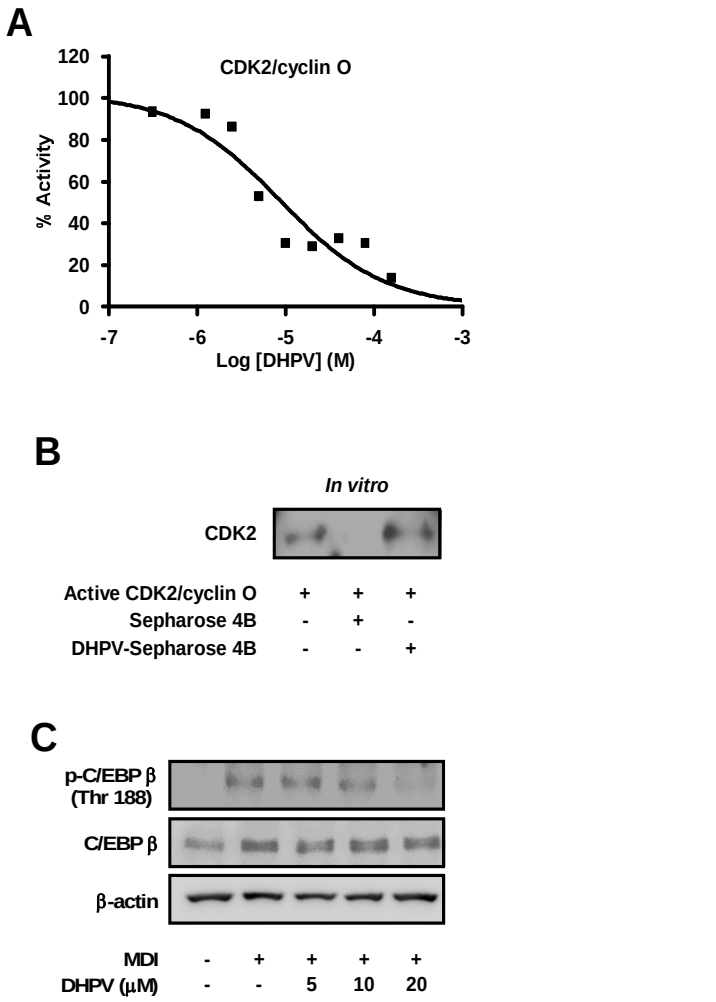
A
CDK2/cyclin O
% Activity
120
100
80
60
40
20
0
-7
-6
-5
-4
-3
Log [DHPV] (M)
B
In vitro
CDK2
Active CDK2/cyclin O + + +
Sepharose 4B - + -
DHPV-Sepharose 4B - - +
C
p-C/EBP β (Thr 188)
C/EBP β
β-actin
MDI - + + + +
DHPV (μM) - - 5 10 20

Figure 5. Effects of DHPV on CDK2/cyclin O activity and binding interaction

(A) DHPV markedly inhibited CDK2/cyclin O activity. (IC_{50}=8.79 μM) (B) DHPV could directly bind to CDK2/cyclin O in vitro. (C) DHPV reduced phosphorylation of C/EBP β in 3T3-L1 preadipocytes. Western data are represented at least three independent experiments.

3.6. DHPV decreased lipid accumulation in 3T3-L1 mature adipocytes.

Next, I evaluated the effects of DHPV on adipocyte hypertrophy. The cells were differentiated by the method as previously described and treated with 10% FBS DMEM supplemented with or without 5, 10, and 20 μM DHPV for 4 days. Lipid contents were quantified with Oil Red O staining. ORO staining result showed that DHPV had the inhibitory effect on intracellular lipid accumulation (Fig. 6A). Then I investigated whether DHPV might regulate the metabolic process of free fatty acids such as lipogenesis. As a result, DHPV suppressed the expression of the SREBP1c, ACC, and FAS which are the lipogenic proteins (Fig. 6B).

The adipocytes are mainly composed of a neutral lipid (Triglyceride) as the most important storage form of fats. Triglycerides are hydrolyzed and release glycerol and fatty acids. Therefore, the

measurement of releasing levels of glycerol and free fatty acids are supposed to reflect lipolysis. However, unlike the glycerol, the free fatty acids can re-use and uptake by adipocytes [41]. Therefore, to evaluate the lipolytic effects of DHPV, I measured the released glycerol contents in the media. But, there was no difference on released glycerol contents (Fig. 6C). This result implied DHPV did not affect on lipolysis and fatty acid oxidation.

To prove that above results were not associated with diminished cell number, I performed MTT assay. As a result, DHPV did not decline cell viability (Fig. 6D).

Figure 6

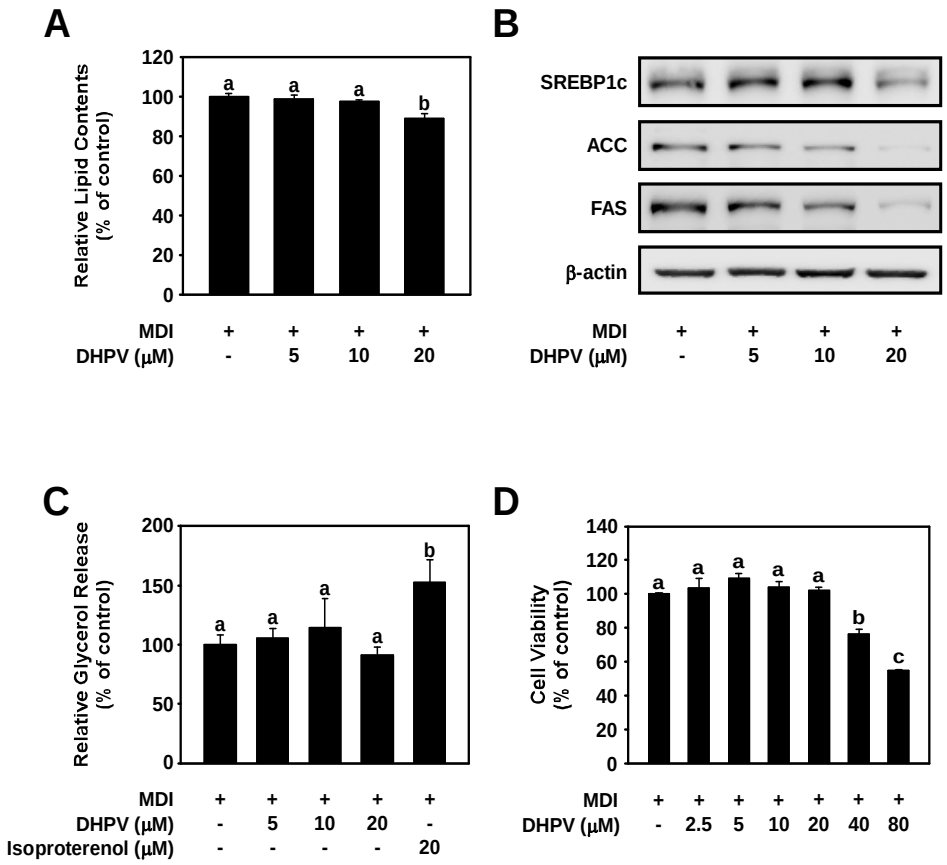
A
Relative Lipid Contents (% of control)
120
100
80
60
40
20
0
a
a
a
b
MDI + + + +
DHPV (μM) - 5 10 20
B
SREBP1c
ACC
FAS
β-actin
MDI + + + +
DHPV (μM) - 5 10 20
C
Relative Glycerol Release (% of control)
200
150
100
50
0
a
a
a
a
b
MDI + + + + +
DHPV (μM) - 5 10 20 -
Isoproterenol (μM) - - - - 20
D
Cell Viability (% of control)
140
120
100
80
60
40
20
0
a
a
a
a
a
b
c
MDI + + + + + + +
DHPV (μM) - 2.5 5 10 20 40 80

Figure 6. Effects of DHPV on lipid accumulation in 3T3-L1 mature adipocytes

(A) DHPV inhibited intracellular lipid accumulation in 3T3-L1 mature adipocytes. (B) DHPV suppressed the expression of lipogenic proteins such as SREBP1c, ACC, and FAS. (C) DHPV had no effect on released glycerol contents. (D) DHPV did not affect on cell viability up to 20 μM. Data were represented as mean ± standard error of mean (SEM) values of at least three independent experiments. To compare the differences among the groups, one-way ANOVA was used with Duncan's multiple range tests. Different letters indicate statistically significant differences at $p < 0.05$

Figure 7

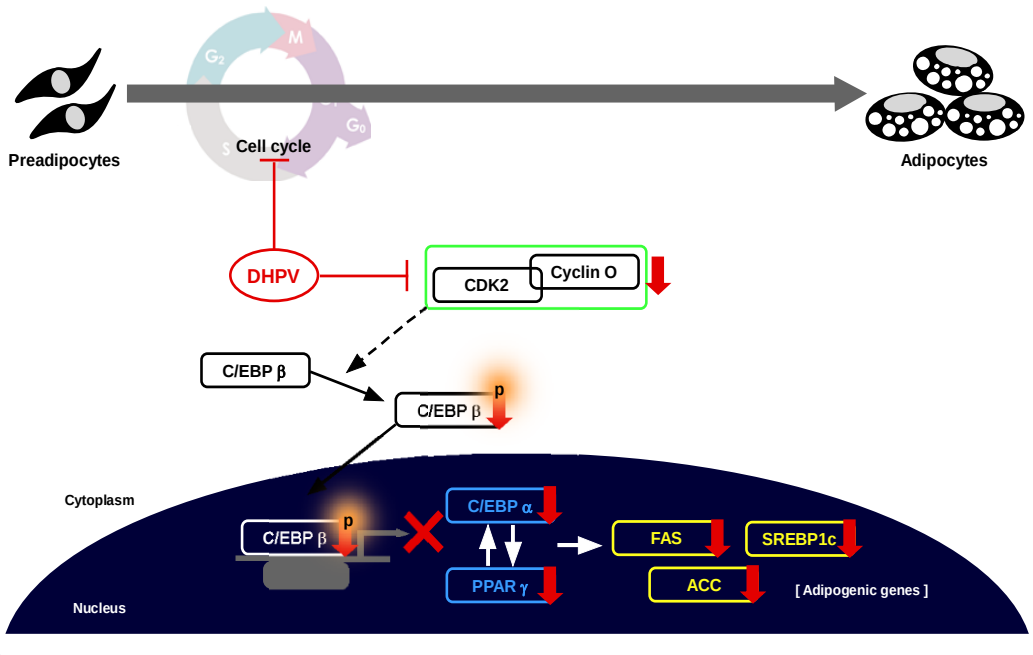

Figure 7. Proposed mechanism of DHPV

Ⅳ. DISCUSSION

Obesity is described as a condition of abnormal or excessive fat accumulation in adipose tissue because of an energy imbalance between calories consumed and expended [38]. When calories consumed is more than expended, excessed calories will store the form of triglyceride then accumulate in adipocytes.

In the present study, I investigated the anti-obesity effects using adipogenesis model. Adipogenesis is the process of cell differentiation by which preadipocytes become adipocytes and it consists of two stages by mitotic clonal expansion and terminal differentiation. I used the 3T3-L1 preadipocytes cell lines to elucidate this model. The 3T3-L1 preadipocytes are a well-established cell line to determine the process of adipogenesis model which can study both hyperplasia (increase in cell number) and hypertrophy (increase in cell size) of

adipocytes [30, 31, 39, 40].

Confluent preadipocytes become growth arrest by contact inhibition and be fixed in G1 stage [41]. When adipocytes are stimulated with a hormonal cocktail consisting of isobutylmethylxanthine (IBMX), dexamethasone, and insulin (MDI), the adipocytes synchronously re-enter the cell cycle then we called this stage, mitotic clonal expansion [42, 43]. DHPV suppressed adipogenesis (Fig. 2) by inhibiting the mitotic clonal expansion stage (Fig. 3). Because DHPV blocked the G1 phase then regulated cell cycle progression (Fig. 4). The cell cycle constitutes four phases as G1, S, G2, and M phase [44, 45]. Cell cycle is adjusted by the several regulatory proteins containing both cyclin-dependent kinases (CDKs) and cyclins. Activation of some CDKs is generally known during the cell cycle: During G1 phase; CDK2, CDK4, and CDK6, During S phase; CDK2, and During G2 and M phase; CDK1 [44]. Therefore, I inferred DHPV

could affect the cell cycle regulatory proteins.

Next, I found to elucidate the direct molecular target of DHPV. I screened the 359 kinases and selected 6 kinases which were inhibited more than 65% by DHPV. And then, I tested various concentrations of DHPV with above kinases, I picked out 5 kinases excluding VRK2 kinase which was false positive because of no detection IC_{50}. Although CDK5/p25 kinase had the lowest IC_{50}, it was pushed back on the priority list. Because CDK5 bond to p25 inhibits PPAR γ pathway by its phosphorylation [46, 47]. Also, mice which ablated CDK5 in adipose tissues worsen insulin resistance [48].

For these reasons, I preferentially chosen CDK2/cyclin O kinase for potential target of DHPV based on these following prerequisites; expression in mouse and adipocytes, regulation to cell cycle, and relation to MDI-induced signaling. I further determined that DHPV played a significant role in modulating CDK2/cyclin O activity

by binding directly (Fig. 5A and B).

According to Figure 4, DHPV regulated cell cycle progression by blocking G1 phase. CDK2 is well-known for regulating G1 and S phase during cell cycle [49-51]. Additionally, Cyclin O is a novel cyclin family protein which is also involved in cell cycle. Cyclin O has been reported as a G1 phase regulated protein whose promoter region contains cis-acting elements. Also, cyclin O protein and mRNA levels increase during G1 phase in vitro [52]. Additionally, cyclin O is been reported to interact with CDK2 then induces the intrinsic apoptosis pathway in lymphoid cells [53].

In mitotic clonal expansion stage, the cells increase in number and require CCAAT/enhancer-binding protein β (C/EBP β) which is stimulated by IBMX. C/EBP β is an important protein to initiate mitotic clonal expansion [54, 55] because it is an essential prerequisite for transcription of master regulator of proteins in terminal differentiation

can be activated [33-37]. C/EBP β sequentially phosphorylates, first, a priming phosphorylation on Thr 188 by MAPK/ERK in G1 phase. Next, C/EBP β undergoes a phosphorylation on Thr 179 or Ser 184 by glycogen synthase kinase 3β (GSK 3β) [33, 56]. After dual phosphorylation, C/EBP β undergoes a conformational change then induces dimerization through its C-terminal leucine zipper domain [57, 58]. As a result, it can bind with DNA and obtain the transactivation capacity. In this process, CDK2 plays a role to retain the phosphorylated (Thr 188) state of C/EBP β during mitotic clonal expansion and thereby progression of terminal differentiation [33]. Therefore, I investigated the protein expression of phosphorylation of C/EBP β, DHPV suppressed the expression of protein which activates the key markers such as C/EBP α and peroxisome proliferator-activated receptor γ (PPAR γ) (Fig. 5C).

I demonstrated that DHPV effectively inhibited adipogenesis

and lipogenesis in vitro model using 3T3-L1 preadipocytes. However, one of the biggest unsolved issue on using fat cells study, it is still remain how it can be applicable on human. Also, adipocytes are active organ which is not only storage organ of energy but also endocrine organ of several hormones. It is considered as cross-talk between adipocytes and other organs [41]. Therefore, it is required that the anti-obesity effects of DHPV in vivo and clinical studies. Also effectively physiological dosage needs to be determined by in vivo animal and clinical case.

In summary, DHPV suppressed MDI-induced adipogenesis in 3T3-L1 preadipocytes by delaying cell cycle progression. DHPV inhibited CDK2/cyclin O activity and thereby, regulated C/EBP β phosphorylation and reduced adipogenic markers. Additionally, DHPV inhibited MDI-induced lipogenesis by suppression lipogenic markers (Fig. 7).

Taken together, these observations implied that DHPV which presents a significant increase in plasma after cocoa consumption, could be a potential compound for the objective assessment of procyanidins in cacao intake that could help to beneficial effects on anti-obesity. Further, it is required how DHPV structurally binds CDK2/cyclin O and regulates its function. And because, there is a possibility that another target interacts with DHPV. Therefore, I need to perform additional studies to determine the other targets.

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Ⅵ. 국문 초록

비만은 체내에 지방조직이 과다한 상태로 비만인구의 증가는 전세계적으로 주요한 문제로 대두되고 있다. 특히, 지방세포의 분화과정은 비만의 원인 중 하나인 지방세포 수의 증가를 결정할 수 있는 중요한 모델로 여겨진다. 카카오는 많은 임상 연구와 전임상 연구에서 항비만 효능을 갖는다고 잘 알려져 있다. 하지만 근본적으로 카카오의 어떠한 성분 때문에 항비만 효능이 나타나는지에 관해서는 알려진 바가 없다. 본 연구에서는 카카오를 섭취한 후 혈중에 가장 높게 존재하는 대사체로 알려진 5-(3', 4'-Dihydroxyphenyl)-γ-valerolactone (DHPV)의 지방세포 분화 및 지방 축적 저해 효능과 그에 관련한 분자기작을 규명하였다. DHPV는 지방세포 분화의 주요 조절 마커인 PPAR γ, C/EBP α, FAS 의 발현을 감소시킴으로써 3T3-L1 지방전구세포의 지방 축적 과정을 억제하였다. 3T3-L1 지방전구세포는 크게 유사분열성 세포 증식 (mitotic clonal expansion)과 최종 분화

(terminal differentiation) 두 과정을 통하여 일어나게 되는데, DHPV는 이 과정 중, 유사분열성 세포 증식을 효과적으로 저해하였으며 세포분열 주기 중 G1기의 진행을 억제하였다. 또한, CDK2/cyclin O의 활성을 저해함으로써, 이의 하위인자이며 지방세포 분화에 중요한 마커인 C/EBP β의 인산화 역시 감소시켰다. 즉, DHPV는 CDK2/cyclin O의 활성을 감소시켜 세포분열을 저해함으로써 최종적으로 3T3-L1 지방전구세포의 분화를 억제함을 확인하였다. 이를 통하여 카카오의 항비만 효능 성분으로서 가능성을 제시하였으며 식품에서 유래한 안전성이 높은 천연물로 항비만을 예방하고 치료하는 물질로 활용할 수 있을 것이다.

주요어: 비만; 지방세포 분화; 지방 축적; 지방전구세포; 5-(3', 4'-Dihydroxyphenyl)-γ-valerolactone (DHPV);

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