

# Silibinin Inhibits Adipogenesis and Induces Apoptosis in 3T3-L1 Adipocytes

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**C/EBP $\beta$  and C/EBP $\delta$  are required for the initiation of adipogenesis and induce the expression of key adipogenic regulators, such as PPAR $\gamma$  and C/EBP $\alpha$ . In the present study, we have examined the effects of silibinin and its possible molecular mechanisms in regulating adipocyte differentiation and expression of C/EBP $\beta$  and C/EBP $\delta$  in the early stage of adipogenesis. Silibinin statistically significantly inhibits intracellular lipid accumulation and the mRNA expression of various genes involved at different stages during adipogenesis. Silibinin also suppresses expression of lipoprotein lipase (LPL), fatty acid binding protein 4 (AP2), and adiponectin in 3T3-L1 adipocytes. Thus, the anti-adipogenic effect of silibinin seems to originate from the ability to inhibit the expression of C/EBP $\beta$  and C/EBP $\delta$ . Furthermore, silibinin decreases cell viability for differentiation period and induces apoptotic cell death through caspase-3 activation.**

**Keywords:** 3T3-L1 adipocytes, anti-adipogenic, obesity, silibinin, *Silybum marianum*

## Introduction

Obesity is a global health problem that is associated with an increased risk of numerous diseases, including diabetes, metabolic syndrome, and cardiovascular disease [1, 2]. Excessive body fat, i.e. an increase in adipose tissue mass, results from adipogenesis and increased deposition of cytoplasmic triglycerides (TGs) [3]. Adipose tissue mass can be reduced by inhibiting *adipogenesis* or by inducing apoptosis of preadipocytes and mature *adipocytes* [4]. Adipogenesis is characterized by hyperplasia (increased number) and hypertrophy (increased size) of adipocytes. Adipogenesis is tightly controlled by several transcription factors, including peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) and CCAAT/enhancer binding proteins (C/EBP $\beta$ , C/EBP $\delta$ , and C/

EBP $\alpha$ ), that are necessary for the expression of many adipogenic genes, including fatty acid synthase (FAS) and adipocyte fatty acid-binding protein (aP2) [5]. C/EBP $\beta$  and C/EBP $\delta$  are expressed in the early phase of adipogenesis and induce proliferation of non-confluent adipocytes. This process, known as mitotic clonal expansion (MCE), is essential for adipocyte differentiation [6, 7]. Understanding these processes is important in developing drugs to control obesity.

Flavonoids have recently attracted considerable interest as potential therapeutics against cancer, obesity, and cardiovascular disease [8, 9]. Among the various flavonoids, silibinin (also known as silybin), extracted from the milk thistle (*Silybum marianum*), has been reported to have anti-oxidative and anti-inflammatory actions [10, 11]. In addition, several studies have demonstrated direct effects of silibinin on adipocyte differentiation, but the mechanism of this action is not well understood [12, 13].

Therefore, in the present study, we have investigated

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the effects of silibinin on adipogenesis and the related molecular mechanisms.

## Materials and Methods

### Chemical reagents

Silibinin, Dulbecco's Modified Eagle's Medium (DMEM), antibiotics (100,000 unit/l penicillin, 100 mg/l streptomycin), fetal bovine serum (FBS), and bovine calf serum (BCS) were purchased from Gibco (USA). Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), indomethacin, and Oil Red O (ORO) reagent were purchased from Sigma-Aldrich (USA) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (USA). A triglyceride quantification kit (#K622-100) was supplied by Biovision Inc. (USA). Trizol reagent was obtained from Invitrogen (USA). Primary antibodies were obtained from the following sources: anti-pro-caspase-3, anti-cleaved-caspase-3, anti-p38, anti-p-p38, and anti-C/EBP $\alpha$  antibodies from Cell Signaling Technology (USA), anti-PPAR $\gamma$  and anti-adiponectin antibodies from Abcam plc (UK), anti- $\beta$ -actin antibody from Santa Cruz Biotechnology (USA).

### Cell culture and differentiation

The 3T3-L1 mouse cell line was purchased from the Korean Cell Line Bank (Korea) and maintained in DMEM with 10% BCS at 37°C in a humidified 5% CO<sub>2</sub> incubator. The 3T3-L1 cells were seeded into six-well plates and differentiated into mature adipocytes. To induce differentiation, 2 days after reaching confluency (designated as day 0), the cells were stimulated with MDI solution (0.5 mM IBMX, 0.25  $\mu$ M DEX, 167 nM insulin, and 100  $\mu$ M indomethacin) in DMEM and 10% FBS for 2 days (from day 0 to day 2). The culture medium was then replaced with DMEM supplemented with 10% FBS and insulin (from day 2 to day 9), which was changed every 2 days. To examine the effect of silibinin on adipogenesis, the cells were cultured with the differentiation medium in the presence or absence of silibinin (7.5 or 75  $\mu$ M).

### Cell viability (MTT assay)

The 3T3-L1 cells were seeded in ninety-six-well plates at a density of  $3 \times 10^3$  cells/well and induced to undergo adipocyte differentiation or not induced. After incuba-

tion, 3T3-L1 preadipocytes and differentiated adipocytes were treated with silibinin (7.5 or 75  $\mu$ M) or were left untreated. At the end of the treatment, the culture medium was removed and replaced with MTT solution (0.5 mg/ml). The cells were then incubated for 3 h at 37°C followed by treatment with isopropyl alcohol (*Duksan* pure chemicals, Korea). The absorbance of each sample was measured at 495 nm.

### Oil Red O staining

For staining, 3T3-L1 cells were treated with silibinin (7.5 and 75  $\mu$ M) for 8 days during adipogenesis or were left untreated. The cells were washed with phosphate-buffered saline (PBS) and fixed with 5% formalin for 1 h at room temperature. The cells were then washed with 60% isopropanol, stained with 0.4% ORO for 10 min at room temperature, washed 4 times with distilled water and photographed with a microscope at 200 $\times$  magnification. Images of 3 random fields from 3 replicate wells were obtained and cells stained with Oil Red O solution was dissolved in isopropanol and then the absorbance was measured at 495 nm.

### Triglyceride colorimetric assay

TG content was determined using a TG Quantification Kit (Bio Vision) according to the manufacturer's instructions. At the end of the treatment, mature 3T3-L1 adipocytes were washed with PBS, homogenized in TG assay buffer containing 5% Nonidet P-40, heated to 80°C for 4 min, centrifuged at 16,000  $\times g$  for 2 min to remove insoluble materials and diluted in dH<sub>2</sub>O. The samples were mixed with a TG reaction mixture and incubated at room temperature for 1 h. Colorimetric intensity was determined at 570 nm.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 3T3-L1 adipocytes using RNAiso Plus reagent (TaKaRa Bio, Japan). RNA was used for complementary DNA (cDNA) synthesis using the Prime-Script RT reagent Kit (TaKaRa Bio, Japan) according to the manufacturer's protocol. PCR amplification of the cDNA products was performed with GT PCR Master Mix (TaKaRa Bio, Japan). Amplification consisted of 35 cycles as follows: denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s, followed by a final 10 min extension at 72°C.

**Table 1. The primer sequences for adipogenic genes.**

Gene name	Accession no.	Forward primer	Reverse primer
aP2	NM_024406	AACACCGAGATTCCTTCAA	TCACGCCTTTCATAACACAT
Adiponectin	NM_009605	ACCTACGACCAGTATCAGGAAAAG	ACTAAGCTGAAAGTGTGTCGACTG
C/EBP $\alpha$	NM_001287523	TTACAACAGGCCAGGTTTCC	GGCTGGCGACATACAGATCA
C/EBP $\beta$	NM_009883	GACTACGCAACACACGTGTAAC	CAAAACCAAAAACATCAACAACCC
C/EBP $\delta$	NM_007679	GATCTGCACGGCCTGTTGTA	CTCCACTGCCACCTGTGTC
LPL	NM_008509	TCCTCTGACATTGCAGGTCTATC	TCACGCCTTTCATAACACAT
PPAR $\gamma$	AB644275	TTTTCAAGGGTGCCAGTTTC	AATCCTTGGCCCTCTGAGAT
$\beta$ -actin	EF095208	GACAACGGCTCCGGCATGTGCAAAG	TTCACGGTTGGCCTTAGGGTTCAG

PCR products were electrophoresed on a 1.2% agarose gel and the gel was stained with ethidium bromide. The sequences of the designed primers are shown in Table 1.  $\beta$ -actin was used as the control gene.

#### Western blot analysis

Cell lysates were prepared using radioimmunoprecipitation assay (RIPA) lysis buffer (Biosesang, Korea). Protein samples were separated on 10–15% SDS-PAGE, transferred onto nitrocellulose membranes, blocked using 5% non-fat skim milk in TBST (10 mM/ Tris pH 8.0, 150 mM/ NaCl, 0.05% Tween 20) and incubated with primary antibodies overnight at 4°C. The membrane was incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The proteins were detected using enhanced chemiluminescence (ECL) reagent (GE Healthcare, UK).

#### Statistical analysis

Data were analyzed using SPSS version 21.0 (IBM Corporation, USA). Statistical comparisons between groups were performed using 1-way ANOVA. Values of  $p < 0.05$  were considered statistically significant.

## Results

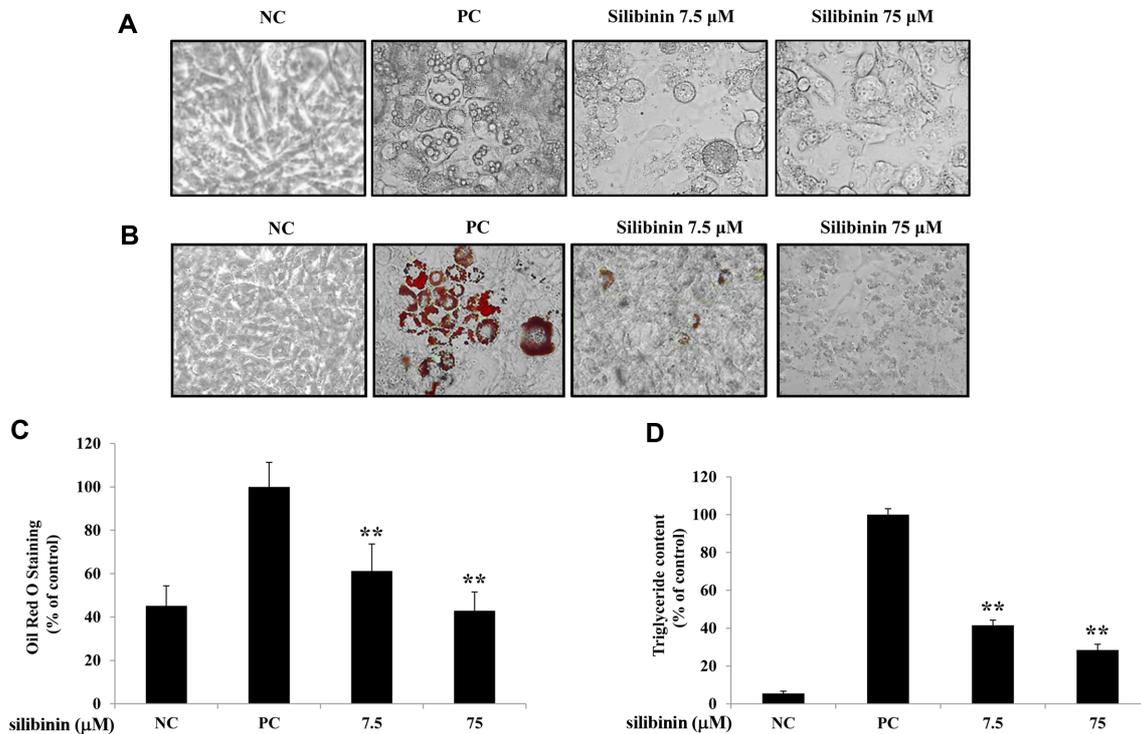
#### Effects of silibinin on differentiation and lipid accumulation of 3T3-L1 cells

Lipid accumulation in adipocytes is one of the hallmarks of adipogenesis [14]. To investigate the effects of silibinin on the differentiation of 3T3-L1 preadipocytes into mature adipocytes, we treated the cells with silibinin at concentrations of 7.5 or 75  $\mu$ M for 8 days and performed ORO staining and a TG colorimetric assay. We

found that silibinin inhibits the differentiation of 3T3-L1 cells in a concentration-dependent manner (Fig. 1A). During differentiation, cells normally change morphology to a more rounded shape and accumulate lipid droplets. Cells treated with silibinin at either concentration exhibited a small and spindle-like morphology. ORO staining revealed that the number of lipid droplets in the differentiated cells treated with silibinin was significantly less than in the untreated control. (Fig. 1B). Quantification of ORO staining demonstrated that the 7.5 and 75  $\mu$ M concentrations of silibinin significantly decreased the amount of lipid accumulation, by 61.2% and 42.8%, respectively (Fig. 1C). In addition, silibinin at concentrations of 7.5 and 75  $\mu$ M markedly decreased intracellular TG levels, by 41.4% and 28.4%, respectively (Fig. 1D). These results show that the lowest silibinin concentration strongly inhibited the differentiation of 3T3-L1 preadipocytes into adipocytes and prevented lipid accumulation.

#### Effect of silibinin on the mRNA and protein expression of adipogenesis-related genes during 3T3-L1 differentiation

Adipocyte differentiation is accompanied by alterations in the expression of various transcriptional factors and adipogenesis-specific genes [14]. To investigate the mechanisms underlying the action of silibinin to suppress differentiation of 3T3-L1 cells, we treated cells that were in the process of differentiation with silibinin for 8 days and performed RT-PCR. Treatment with silibinin at concentrations of either 7.5 or 75  $\mu$ M significantly reduced mRNA expression of transcription factors PPAR, C/EBP $\alpha$ , and C/EBP $\delta$  and also decreased the expression of adipocyte-specific genes, fatty acid-binding protein 4 (aP2), lipoprotein lipase (LPL), and adiponectin.



**Fig. 1. Effects of silibinin on the differentiation and lipid accumulation of 3T3-L1 cells.** 3T3-L1 preadipocytes were treated with silibinin (7.5 or 75  $\mu\text{M}$ ) for 8 days or were untreated. (A) After 8-days, the differentiated cells were photographed with a microscope at 400 $\times$  magnification. (B) Intracellular lipid droplets were stained by ORO solution and observed at 200 $\times$  magnification. (C) The ORO stained cells were measured as absorbance at 495 nm. (D) Intracellular triglyceride concentrations measured as absorbance at 570 nm. The preadipocytes were called negative control (NC) and fully differentiated adipocytes were called positive control (PC). PC were treated with the same amount of DMSO in place of silibinin. *P* values of <0.01 (\*\*) or <0.05 (\*). Bars represent mean  $\pm$  SD from three independent experiments.

The mRNA levels of *C/EBP $\beta$*  were not affected (Fig. 2). Thus, *PPAR*, *C/EBP $\alpha$* , and *adiponectin* were selected for subsequent western blotting studies. The results of the western blot analysis were similar to the RT-PCR. These results suggest that the inhibitory effect of silibinin on mRNA and protein expression was maintained until day 9, the termination stage of 3T3-L1 differentiation.

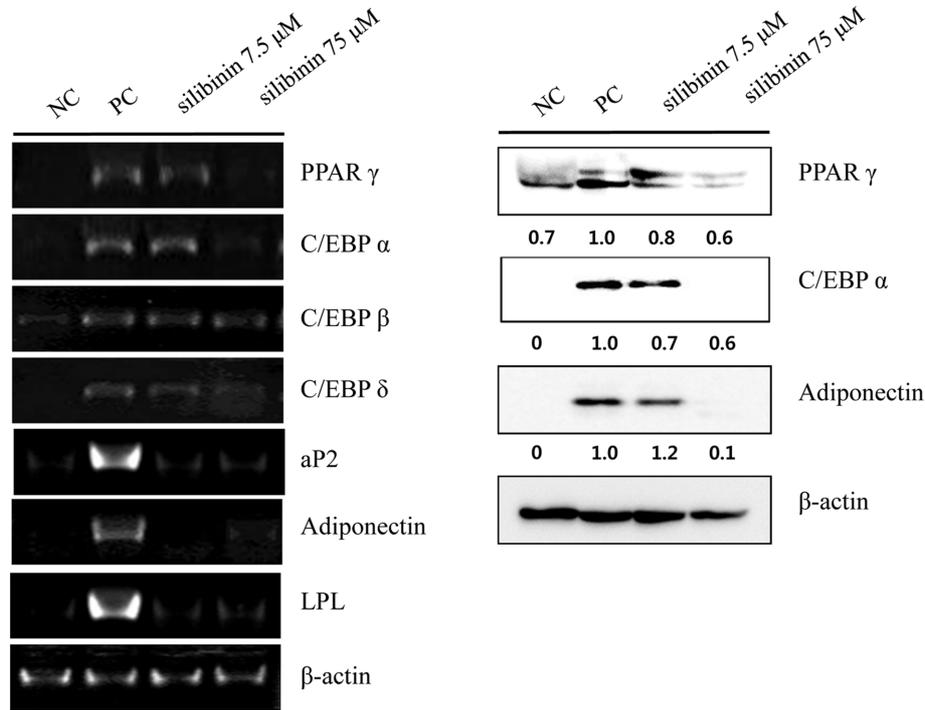
#### Effects of silibinin on mRNA expression of the transcription factors that expressed in the early stage of adipogenesis

*C/EBP $\beta$*  and *C/EBP $\delta$*  are critical determinants for the early stage of adipocyte differentiation and regulate the expression of cell type-specific genes [15, 16]. We therefore examined the effect of silibinin on mRNA expression levels of the early adipogenic transcription factors *C/EBP $\beta$*  and *C/EBP $\delta$*  in the presence or absence of silibinin (7.5 or 75  $\mu\text{M}$ ) at different time points during differentiating days 1–3. When cells were treated with silibinin,

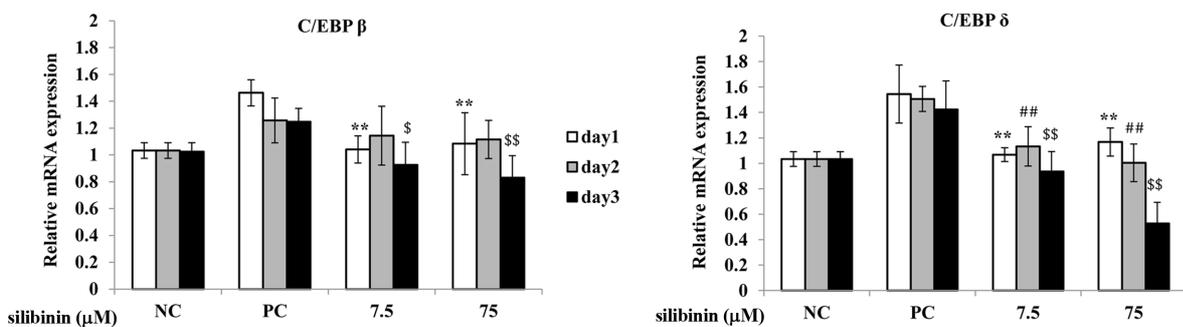
*C/EBP $\beta$*  and *C/EBP $\delta$*  were decreased in a concentration- and time-dependent manner (Fig. 3). These results suggest that inhibition of adipocyte differentiation by silibinin may begin in the early stage of adipocyte differentiation.

#### Silibinin showed cytotoxic effects through induction of apoptosis in differentiating adipocytes

We performed an MTT assay to examine the effects of silibinin on the viability of 3T3-L1 preadipocytes or differentiated adipocytes. Silibinin (7.5 or 75  $\mu\text{M}$ ) had no significant effect on the viability and proliferation of 3T3-L1 preadipocytes (Fig. 4A). Additionally, the cytotoxicity of silibinin on differentiated adipocytes was assessed with 3T3-L1 cells induced to differentiate into mature adipocytes. Treatment with silibinin at concentrations of 75  $\mu\text{M}$  at all stages (0–8 days) or at late stages (4–8 days) of differentiation significantly decreased cell



**Fig. 2. Effects of silibinin on mRNA and protein expression of adipogenesis-related genes in 3T3-L1 adipocytes.** 3T3-L1 preadipocytes cells were with silibinin (7.5 or 75  $\mu\text{M}$ ) for 8 days during differentiation or were untreated. Total RNA and protein was isolated from 3T3-L1 adipocytes at days 8 and confirmed the expression of adipogenic-related genes by RT-PCR and western blot analysis. Representative images for RT-PCR and western blot are shown on the left and right, respectively. *P* values of  $<0.01$  (\*\*) or  $<0.05$  (\*). Bars represent mean  $\pm$  SD from three independent experiments.

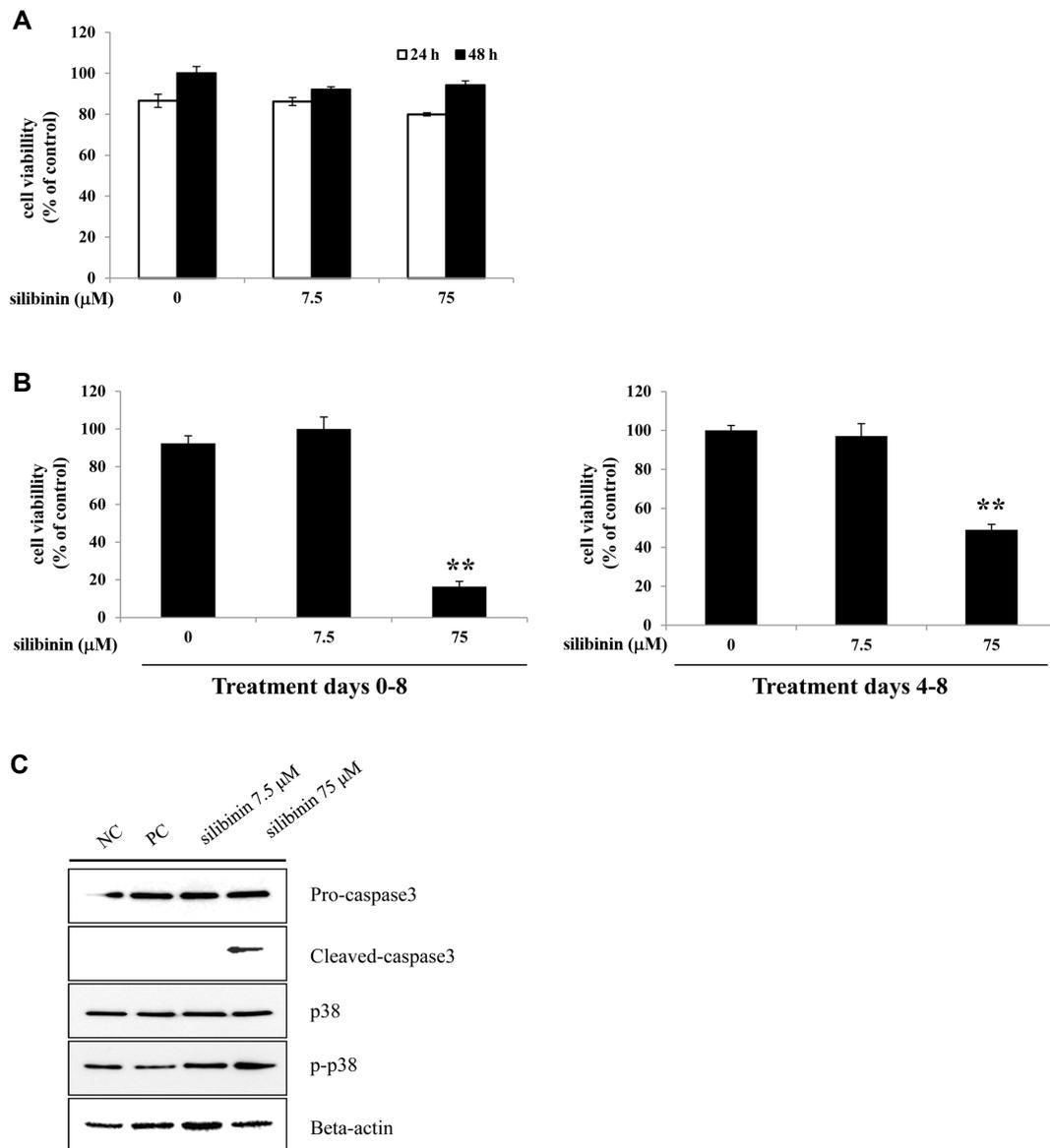


**Fig. 3. Effect of silibinin on the mRNA expression of C/EBP $\beta$  and C/EBP $\delta$  at the early stages of adipogenesis.** 3T3-L1 preadipocytes cells were treated with silibinin (7.5 and 75  $\mu\text{M}$ ) for 3 days or were left untreated. Total RNA was isolated from 3T3-L1 adipocytes at days 1, 2, and 3 during differentiation. The mRNA expression of C/EBP $\beta$  and C/EBP $\delta$  were normalized to  $\beta$ -actin and shown as relative expression compared to the undifferentiated control group (NC) at all times.  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) at day 1,  $p < 0.05$  (#),  $p < 0.01$  (##) at day 2 and  $p < 0.05$  (\$),  $p < 0.01$  (\$\$) at day 3 compared with untreated adipocytes at each gene expression. Bars represent mean  $\pm$  SD from three independent experiments.

viability (Fig. 4B). Treatment with silibinin at all stages had a greater cytotoxic effect than just treatment at late stages.

We hypothesized that cytotoxic effects of silibinin was

predominantly due to apoptosis in differentiating adipocytes. To test this hypothesis, we examined the protein expression of apoptotic makers after the treatment of silibinin with MDI solution for 24 h. Caspase-3 is an



**Fig. 4. Effects of silibinin on cell viability of 3T3-L1 preadipocytes and differentiated adipocytes.** 3T3-L1 preadipocytes were treated with concentrations (7.5 or 75  $\mu\text{M}$ ) of silibinin for 24 and 48 h or were left untreated. (A) Cell viability was determined by MTT assay and measured as absorbance at 495 nm. (B) 3T3-L1 preadipocytes were differentiated into adipocytes and treated with silibinin (7.5 or 75  $\mu\text{M}$ ) for the entire differentiation period (from day 0 to day 8) or after induction of differentiation (from day 4 to day 8). (C) 3T3-L1 cells were induced to differentiate into adipocytes with treatment of silibinin (7.5 or 75  $\mu\text{M}$ ) for 24 h. Protein expression of pro-caspase-3, cleaved-caspase-3, p38, p-p38, and beta-actin were analyzed by western blot. An equal amount of loading was confirmed using  $\beta$ -actin. *P* values of <0.01 (\*\*\*) or <0.05 (\*). Bars represent mean  $\pm$  SD from three independent experiments.

effector of apoptosis and the activation of caspase-3 generally leads to cleavage of cytoplasmic [17]. P38 mitogen-activated protein kinase (p38) is activated and related with cleavage of caspase-3 during apoptosis by a number of stimuli [18]. We observed that cleaved-caspase-3 and

p-p38 expression were significantly increased in silibinin treated cell (Fig. 4C). Total p38 and pro-caspase-3 expression were not changed under the same conditions. These results strongly imply that silibinin induces apoptosis of 3T3-L1 adipocytes during adipogenesis.

## Discussion

Adipogenesis and excessive fat accumulation in white adipose tissues are caused by excess energy intake and lack of activity and are the key features of obesity [19]. Anti-obesity drug development has focused on synthetic chemicals such as orlistat or sibutramine. However, because of the severe side effects of such chemicals, recent studies on anti-obesity agents have focused on natural compounds [14]. Silibinin, a natural flavonoid, has been suggested to exert anti-oxidative and anti-cancer effects and recent studies reported its anti-obesity actions on 3T3-L1 adipocytes [20, 21].

In this study, we have shown that silibinin suppressed the differentiation of 3T3-L1 preadipocytes into adipocytes. These observations are similar to those reported by Park *et al.* [12]. We have further elucidated the effect of silibinin at different time periods during adipocyte differentiation. We found that treatment of 3T3-L1 adipocytes with silibinin significantly decreased lipid accumulation and triglyceride levels in a concentration-dependent manner as measured by ORO staining and TG assay. To initiate the differentiation program, growth-arrested post-confluent 3T3-L1 preadipocytes were treated with hormonal agents [7, 22]. This treatment causes the preadipocytes to re-enter the cell cycle and undergo two rounds of division, a process referred to as MCE [23]. MCE is coordinated by dramatic induction of C/EBP $\beta$  and C/EBP $\delta$ . These transcription factors promote the expression of PPAR $\gamma$  and C/EBP $\alpha$ , the major transcription factors involved in adipocyte differentiation [1].

Differentiation of preadipocytes into mature adipocytes is mediated by consecutive activation of a complex transcriptional cascade and involves several stages. Therefore, we examined C/EBP $\beta$  and C/EBP $\delta$  mRNA expression after exposure to concentrations of silibinin at the early stage of differentiation (day 1–3) and found that silibinin down-regulated C/EBP $\beta$  and C/EBP $\delta$  expression at all times in a concentration- and time-dependent manner.

Furthermore, silibinin significantly reduced the expression of PPAR $\gamma$ , C/EBP $\alpha$  and adipogenic-related genes, such as aP2, LPL and adiponectin, which are commonly used as adipocyte markers at terminal stage of differentiation (day 9). These results suggest that the inhibited expression of PPAR $\gamma$  and C/EBP $\alpha$  by silibinin

is likely to be a consequence of decreased C/EBP $\beta$  and C/EBP $\delta$  at the early stage of differentiation.

Adipocyte apoptosis contributes to reduced adipose tissue mass, which indicate that cell-specific apoptotic events was able to prevent or treat obesity [24]. We confirmed that silibinin showed cytotoxic effect through apoptosis in differentiating adipocytes but does not affect preadipocyte survival. Our study clearly demonstrates the anti-adipogenesis effect of silibinin in differentiation of 3T3-L1 cells.

In summary, our results demonstrate that silibinin suppresses differentiation of 3T3-L1 cells by modulating the expression of central transcription factors and various adipogenesis-related genes at the early stage of differentiation and induces apoptosis in differentiating 3T3-L1 cells. Further research to elucidate the therapeutic role of silibinin in vivo in an obesity animal model is warranted.

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## 국문초록

### Silibinin의 지방세포분화 억제 및 세포사멸 유도 효과

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CCAAT/enhancer-binding protein beta, delta (C/EBPβ, δ)는 지방세포분화 과정의 초기에 필수적으로 요구되며 지방생성 주요 조절인자인 proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein-alpha (C/EBPα)의 발현을 유도한다. 본 연구에서는 silibinin의 지방세포 분화 억제 효과 및 이러한 효과가 지방세포 분화초기에 C/EBPβ 및 C/EBPδ의 발현 조절을 통해 일어난다는 것을 확인하였다. Silibinin은 지방세포 내 지질축적을 억제하고 세포분화 과정 동안 관여하는 다양한 유전자의 mRNA 발현을 억제하였다. 또한 lipoprotein lipase (LPL), fatty acid binding protein 4 (AP2) 및 adiponectin과 같은 지방세포 분화 관련 유전자의 발현을 억제시켰다. 따라서, Silibinin의 지방세포 분화 억제효과는 C/EBPβ 및 C/EBPδ의 발현 억제에 의한 것으로 보인다. 더불어, Silibinin은 caspase-3 활성을 통해 분화하는 세포에 특이적으로 세포사멸을 유도하는 것을 확인하였다.