Silibinin attenuates adipogenesis in 3T3-L1 preadipocytes through a potential upregulation of the insig pathway

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Abstract. In the past few decades, the use of silibinin, a plant flavonoid extracted from the milk thistle, as a hepatoprotective and chemopreventive agent has gained much attention. In this study, we investigated the effects of silibinin on adipogenesis. Treatment with silibinin suppressed terminal differentiation of 3T3-L1 cells into adipocytes as evidenced by Oil red O staining and TG assay results. Real-time RT-PCR analysis revealed that silibinin decreased the expression of adipogenesis-related genes such as CAAT/enhancer binding protein-α, fatty acid synthase, sterol response element binding protein 1c, adipocyte-specific lipid binding protein, peroxisome proliferator-activated receptor γ and lipoprotein lipase, and increased the expression of preadipocyte factor-1, a preadipocyte marker gene. The anti-adipogenic effect of silibinin was associated with the up-regulation of insig-1 and insig-2. Collectively, these results suggest that silibinin inhibits adipocyte differentiation through a potential up-regulation of insig-1 and insig-2 at an early phase in adipocyte differentiation.

Introduction

Obesity is a disorder that results from excess white adipose tissue, which can be attributed to both adipocyte hyperplasia and hypertrophy. Hypertrophy is due to excess triacylglyceride (TG) accumulation in the adipocytes, whereas hyperplasia results from the recruitment of new adipocytes from preadipocytes in adipose tissue. The development of hyperplastic adipose tissue is associated with the most severe forms of obesity and has the poorest prognosis for treatment (1,2). The differentiated cells exhibit many of the morphological and biochemical characteristics of adipocytes found in white adipose tissue, such as TG accumulation, insulin-regulated metabolism and expression of characteristic adipocyte genes, such as CAAT/enhancer binding protein α (C/EBPα), peroxisome proliferator-activated receptor γ (PPARγ), fatty acid synthase (FAS), lipoprotein lipase (LPL), sterol response element binding protein (SREBP) and adipocyte-specific lipid binding protein (aP2) (3,4). Of these, PPARγ is a master transcription factor for adipogenesis that stimulates the expression of many of the genes necessary for adipogenesis (5).

Feeding normal rats a high fat diet increases the insulin-induced gene 1 (*insig-1*) mRNA levels in white adipose tissue (6). Insig-1 binds to SREBP cleavage activating protein (SCAP), which escorts SREBP from the endoplasmic reticulum to the golgi where the SREBP is proteolytically processed to yield an active transcription factor. The binding of SCAP by insig-1 effectively prevents SREBP activation and thus blocks its action on gene transcription (7,8). It has also been demonstrated that insig-1 restricts lipogenesis in mature adipocytes and blocks differentiation in preadipocytes (9). Insig-2 is a close homolog of insig-1, and also prevents the proteolytic processing of SREBP (7). SREBP1c, an SREBP isoform, activates PPARγ by inducing its expression as well as by promoting the production of an endogenous PPARγ ligand (10).

Silibinin, a plant flavonoid, is the major element extracted from the milk thistle (*Silybum marianum*). To date, studies conducted on both silibinin and its cruder form, known as silymarin, have focused on the hepatoprotective and chemopreventive effects (11). To our knowledge, the antiobesity effects of silibinin have not yet been reported. In the present study, we investigated the feasibility of using silibinin to prevent adipose conversion.

Materials and methods

Cell culture. 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml of penicillin in a humidified atmosphere of 5% CO_2/95% air at 37˚C. For the 3T3-L1 differentiation experiments, confluent cells were treated with differentiation medium (MDI: DMEM, 10% FBS, 1 μM dexamethasone, 10 μg/ml insulin and 0.5 mM

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Table I. Sequences and accession numbers for primers (forward, FOR and reverse, REV) used in real-time RT-PCR.

Gene	Sequences for primers	Accession No.
PPARγ	FOR: GAAAGACAACGGACAAATCACC REV: GGGGGTGATATGTTTGAACTTG	NM_011146
aP2	FOR: AGCCTTTCTCACCTGGAAGA REV: TTGTGGCAAAGCCCACTC	NM_024406
FAS	FOR: TGATGTGGAACACAGCAAGG REV: GGCTGTGGTGACTCTTAGTGATAA	NM_007988
LPL	FOR: GGACGGTAACGGGAATGTATGA REV: TGACATTGGAGTCAGGTTCTCTCT	NM_008509
HSL	FOR: GGAGCACTACAAACGCAACGA REV: TCGGCCACCGGTAAAGAG	NM_010719
C/EBP-α	FOR: TTGTTTGGCTTTATCTCGGC REV: CCAAGAAGTCGGTGGACAAG	NM_007678
SREBP1c	FOR: GGTTTTGAACGACATCGAAGA REV: CGGGAAGTCACTGTCTTGGT	NM_011480
Pref-1	FOR: GCTGGGACGGGAAATTCTG REV: TCCAGGTCCACGCAAGTTC	NM_010052
Insig-1	FOR: TGTGGTTCTCCCAGGTGACT REV: TAGCCACCATCTTCTCCTCC	NM_153526
Insig-2	FOR: TGAAGCAGACCAATGTTTCAA REV: GGTGAACTGGGGGTCTCC	NM_133748
GAPDH	FOR:CGTCCCGTAGACAAAATGGT REV:TTGATGGCAACAATCTCCAC	NM_008084

isobutylmethylxanthine) for 2 days. The medium was then replaced with standard medium containing 5 μg/ml insulin and changed every other day for the following 4 days. Cellular TG content was assayed using a TG assay kit (Sigma, St. Louis, MO) as described previously (12).

Oil red O staining. Cells were washed twice with PBS and fixed with 10% formaldehyde for 2 h at room temperature. After washing once with 60% isopropanol, the cells were stained for 1 h at room temperature with filtered Oil red O/60% isopropanol solution. The cells were then washed once with 60% isopropanol, and once with distilled water. The red-stained adipocytes were observed by light microscopy.

RNA isolation and real-time RT-PCR. Total RNA was extracted from 3T3-L1 adipocytes using a TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was then precipitated with isopropanol and dissolved in diethylpyrocarbonate-treated distilled water. First-strand cDNA was generated with the oligo dT-adaptor primers by reverse transcriptase (Takara, Japan). Specific primers (Table I) were designed using primer express software (Applied Biosynthesis, Foster City, CA). GAPDH was used as the invariant control. The real-time RT-PCR reaction, which contained a final volume of 10 μl, 10 ng of reverse transcribed total RNA, 200 nM of forward and reverse primers and PCR master mixtures, was performed in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). All reactions were conducted in triplicate.

Statistical analysis. All results were expressed as the mean ± the SEM, and the difference between groups was calculated using the Student's t-test. Differences with a $p<0.05$ were considered to be statistically significant.

Results

Effects of silibinin on differentiation of 3T3-L1 cells. We first examined the effects of silibinin on adipocyte differentiation. Upon induction of adipogenesis with MDI, the massive accumulation of cytosolic TG was observed in control cells as visualized by Oil red O staining. When treated with silibinin during the first 2 days of differentiation, preadipocytes failed to differentiate into adipocytes and still retained their spindle-like features characteristic of preadipocytes. A suppressive effect on adipocyte differentiation was evident with as little as 5 μM silibinin and increased in a manner directly related to silibinin concentration (Fig. 1A). Additionally, cellular TG contents were measured to confirm the data from the Oil red O staining. As shown in Fig. 1B,

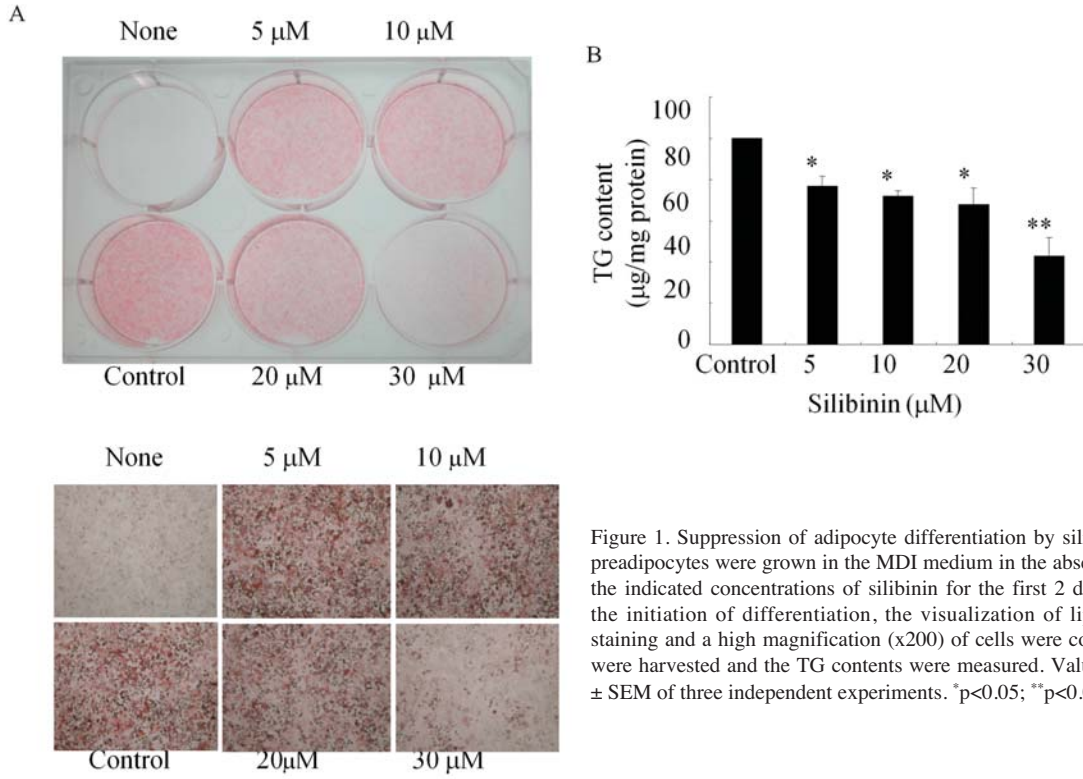

Figure 1. Suppression of adipocyte differentiation by silibinin. (A) 3T3-L1 preadipocytes were grown in the MDI medium in the absence or presence of the indicated concentrations of silibinin for the first 2 days. Six days after the initiation of differentiation, the visualization of lipids by Oil red O staining and a high magnification (x200) of cells were conducted. (B) Cells were harvested and the TG contents were measured. Values represent mean ± SEM of three independent experiments. *p<0.05; **p<0.01 vs. control.

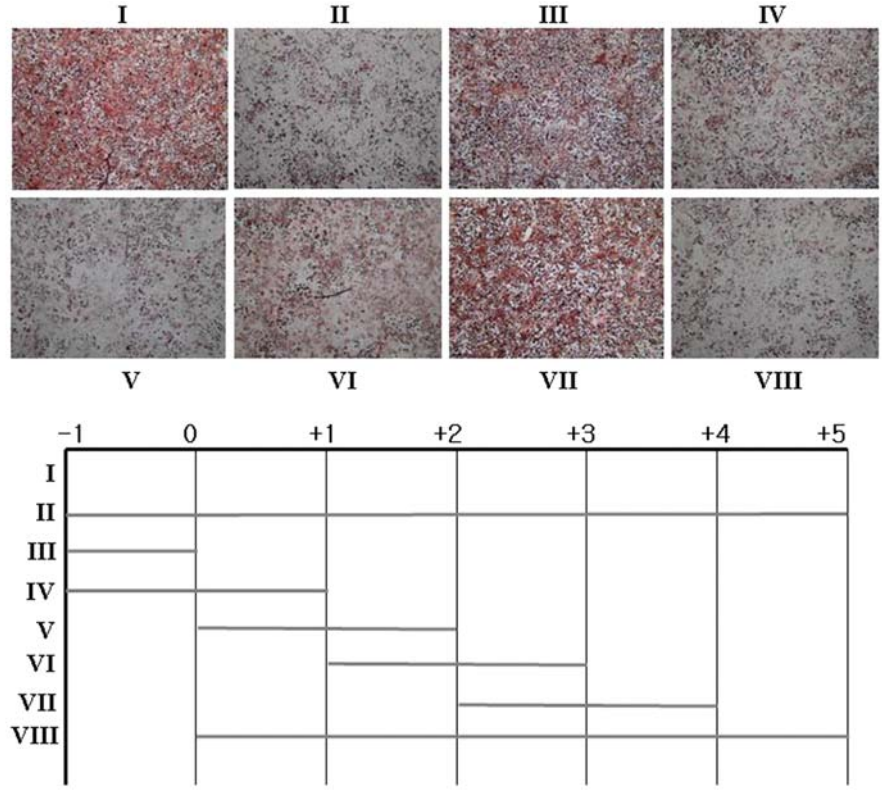

Figure 2. Time-dependent suppression of adipocyte differentiation by silibinin. Differentiating 3T3-L1 preadipocytes were treated with 30 μM silibinin for various time periods and stained with Oil red O. Bars indicate the duration of the silibinin treatment. Representative data from three separate experiments are shown.

TG contents were significantly reduced in silibinin-treated 3T3-L1 cells. Silibinin alone did not affect the viability at the concentrations tested (data not shown).

Next, we treated the 3T3-L1 cells with different time-based protocols to determine the critical periods during which silibinin suppresses TG accumulation. As shown in Fig. 2,

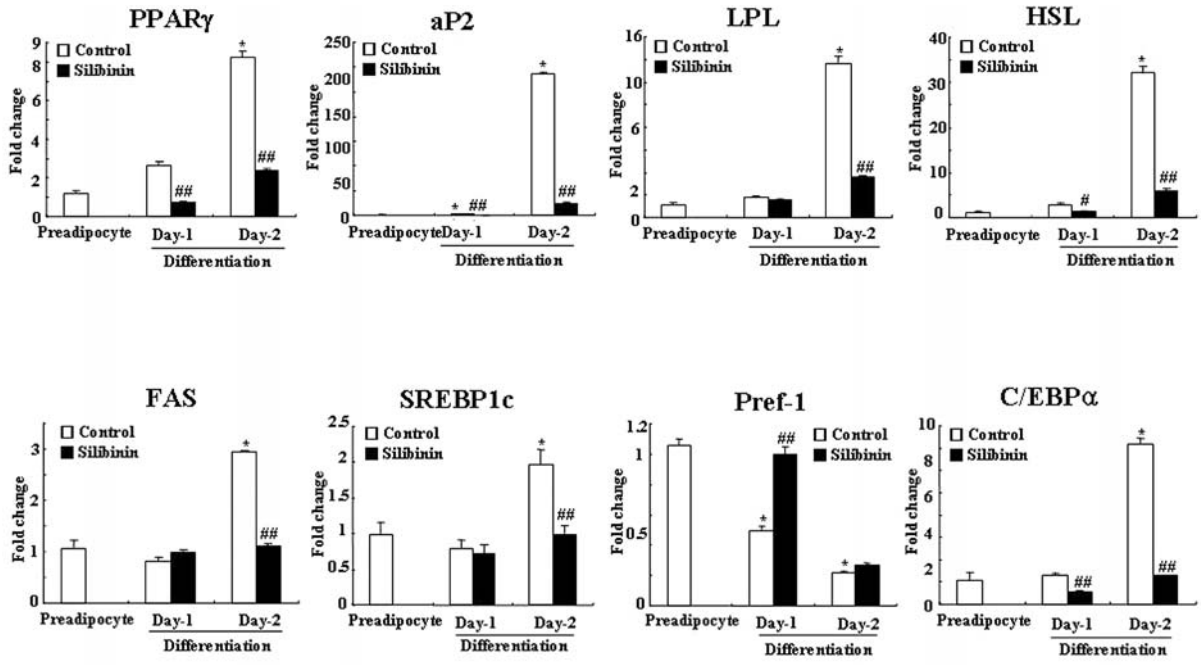

Figure 3. Real-time RT-PCR analysis of adipocyte differentiation marker genes. Differentiating 3T3-L1 preadipocytes were treated with 30 μM silibinin for 1 or 2 days. RNA was isolated and adipogenic gene expression was measured by real-time RT-PCR. Each value is expressed as the mean ± SEM of three independent experiments. $^{*}p<0.05$, vs. preadipocytes; $^{\#}p<0.05$; $^{\#\#}p<0.01$ vs. control.

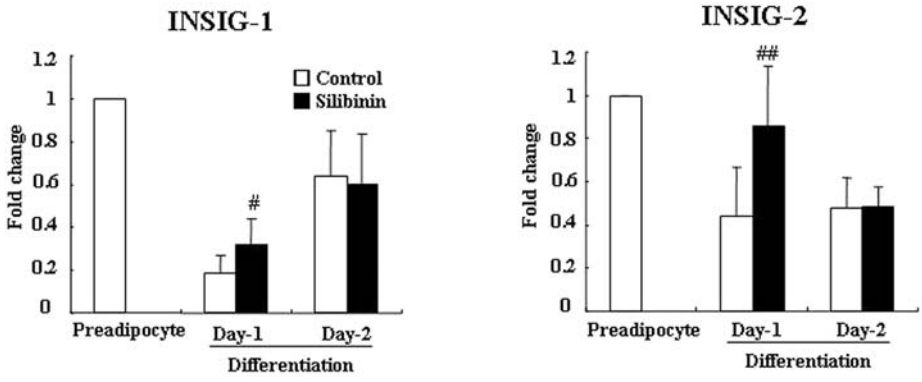

Figure 4. Changes in insig-1 and insig-2 mRNA expression by silibinin. Experimental procedures for the quantification of insig-1 and insig-2 mRNA were the same as described in the legend of Fig. 3. The results of three independent experiments are expressed as the mean ± the SEM. $^{*}p<0.05$, vs. preadipocytes; $^{\#}p<0.05$; $^{\#\#}p<0.01$ vs. control.

treatment with 30 μM silibinin for the first 2 days of differentiation induction was sufficient to suppress MDI-induced adipocyte differentiation and TG accumulation. However, treatment of 3T3-L1 cells with silibinin subsequent to the initial 2 days of exposure to MDI showed little effect, suggesting that silibinin itself suppresses adipocyte differentiation, and has no effect on the later lipid accumulation stage.

Effect of silibinin on expression of adipocyte-specific genes. Total RNA was extracted on the 1st or 2nd day of differentiation and the expression profiles of adipocyte genes were investigated by quantitative real-time RT-PCR (Fig. 3). In the silibinin-treated cells, the expression levels of C/EBP-α, PPARγ, aP2, FAS, LPL and SREBP1c mRNA were several times lower than that of the controls, whereas the expression of pref-1, a preadipocyte marker that normally disappears during adipocyte maturation, increased significantly compared to the control.

Increase of insig-1 and insig-2 expression in silibinin-treated 3T3-L1 cells. Insig-1 and insig-2 are crucial for SREBP-dependent gene transcription and the regulation of adipocyte differentiation (7). We therefore investigated the changes of insig-1 and insig-2 mRNA expression levels in silibinin-treated 3T3-L1 cells. As shown in Fig. 4, mRNA levels of insig-1 and insig-2 increased significantly in the silibinin-treated cells.

Discussion

This study demonstrates that silibinin inhibits the adipose conversion induced by MDI treatment of 3T3-L1 cells. When 3T3-L1 cells are differentiated in the presence of silibinin,

adipogenesis, as measured by lipid accumulation and induction of several adipocyte-specific marker proteins, is severely inhibited. We have further demonstrated that silibinin inhibited adipogenesis through the induction of insig-1 and insig-2 expression.

The time frame during which silibinin exerts its effect was narrow; 3T3-L1 cells treated with silibinin for the first 2 days suppressed MDI-induced adipocyte differentiation, but cells treated with silibinin at a later stage were not different from control cells. A number of transcription factors have been identified that directly influence adipogenic differentiation. Among these, C/EBPs, PPARγ and SREBP1c have been reported to play a major role (10). Induction of preadipocyte differentiation produces a transient increase in the expression of C/EBPß and C/EBPδ, both of which are involved in the activation of PPARγ and C/EBPα (10,13). We therefore characterized the adipocyte gene expression profliles involved in the suppression of adipogenesis by silibinin. Silibinin treatment in an early stage of differentiation caused a significant inhibition of C/EBP-α and PPARγ expression, which would be expected to subsequently reduce late differentiation gene expression and thereby inhibit further differentiation. The results of TG measurement also suggest that silibinin suppresses adipogenesis. Therefore, the anti-adipogenic effect of silibinin in our study is mediated through the down-regulation of the adipogenic program.

The study next examined which signal pathway silibinin uses to regulate the adipogenesis. It is very possible that silibinin works by inducing a regulator that acts as a repressor of adipogenic gene expression. In support of this hypothesis, we proposed that insig proteins were possible targets of silibinin. Previous studies have demonstrated that insig proteins down-regulate the SREBP pathway, and consequently regulate cholesterol and fatty acid metabolism (7,8). In addition, Li *et al* (9) has demonstrated that insig-1 inhibits adipogenesis in preadipocytes. A recent study done by Krapivner *et al* (14) suggests the relative importance of insig-2 in adipose metabolism. According to their study, insig-1 is expressed predominantly in liver tissue, whereas insig-2 is highly expressed in extrahepatic tissues including adipose tissue. In addition, the change in insig-2 expression during adipocyte differentiation is greater than that of insig-1 (9,14). In agreement with these studies, we found that the insig-2 mRNA expression was higher than insig-1 mRNA expression in silibinin-treated 3T3-L1 cells. Taken together, silibinin treatment increases the expression of insig proteins, which might reduce the concentration of nuclear SREBP1c and reduce the expression of genes related to the adipogenic program. The molecular mechanism by which silibinin enhances the expression of insig proteins remains obscure. Further studies are needed to elucidate the exact mechanism.

Silibinin treatment decreases adipogenic differentiation in a dose-dependent manner. This effect was observed with as little as 5 μM silibinin, and 30 μM silibinin almost completely inhibits adipogenic differentiation. Furthermore, the concentrations of silibinin tested in our study were lower than those used in other studies; 100 μM was used for primary isolated hepatocytes (15), 300 μM concentration for MCF-7 cells (16), and 500 μM for primary isolated cardiomyocytes (17). MTT data and morphological observation after silibinin treatment demonstrated that the cells were viable. Thus, our results suggest that the present observations are not due to the non-specific toxic effects of silibinin. Additional *in vivo* studies are needed to establish if there is a causal association between silibinin treatment and body fat deposition.

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