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Vitisin A inhibits adipocyte differentiation through cell cycle arrest in 3T3-L1 cells

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ABSTRACT

Inhibition of adipocyte differentiation is one approach among the anti-obesity strategies. This study demonstrates that vitisin A, a resveratrol tetramer, inhibits adipocyte differentiation most effectively of 18 stilbenes tested. Fat accumulation and PPARγ expression were decreased by vitisin A in a dose-dependent manner. Vitisin A significantly inhibited preadipocyte proliferation and consequent differentiation within the first 2 days of treatment, indicating that the anti-adipogenic effect of vitisin A was derived from anti-proliferation. Based on cell cycle analysis, vitisin A blocked the cell cycle at the G1-S phase transition, causing cells to remain in the preadipocyte state. Vitisin A increased p21 expression, while the Rb phosphorylation level was reduced. Therefore, vitisin A seems to induce G1 arrest through p21- and consequent Rb-dependent suppression of transcription. On the other hand, ERK and Akt signaling pathways were not involved in the anti-mitotic regulation by vitisin A. Taken together, these results suggest that vitisin A inhibits adipocyte differentiation through preadipocyte cell cycle arrest.

Obesity is a strong risk factor for metabolic disorders, such as type II diabetes, hypertension, and coronary heart disease, and has become a worldwide public health threat. Adipocyte differentiation has often been a target of anti-obesity strategies, because obesity is caused not only by hypertrophy of adipocytes, but also by adipocyte hyperplasia [1]. Adipocyte hyperplasia is mimicked *in vitro* by the 3T3-L1 adipocyte differentiation system. The entire adipogenic process consists of the preadipocyte proliferation and their differentiation into mature adipocytes, and this proliferation has proven to be a prerequisite for adipogenesis [2,3]. Upon receiving the appropriate combination of adipogenic signals, growth-arrested 3T3-L1 preadipocytes initiate mitotic clonal expansion and re-enter the cell cycle progression [4]. After withdrawal from the cell cycle, preadipocytes initiate expression of adipocyte-specific genes [4–6]. The peroxisome proliferator-activated receptor (PPAR) γ and CCAAT/enhancer-binding protein (C/EBP) α play essential roles in adipogenic differentiation through transcription of various genes responsible for fat transport and accumulation [4–6].

Inedible parts (stems and roots) of the *Vitis* genus (grape) have been widely used as traditional therapeutic agents possibly because they contain various phytochemicals, particularly resveratrol. Although resveratrol derivatives are also abundant in the stems and roots of *Vitis* [7], their health benefits have been little studied compared to resveratrol. Indeed, among its derivatives, vitisin A, a resveratrol tetramer abounding in the stembark of *Vitis*, has been reported to have anti-inflammatory, neuroprotective, and anti-cholesterolemic activities [8–11]. In a preliminary study (unpublished data), the authors found that resveratrol oligomers isolated from the stembark of *Vitis vinifera* had an inhibitory effect on adipocyte differentiation. In this study, the 18 known stilbene compounds were evaluated to identify the most effective, including resveratrol and vitisin A. This study demonstrated that vitisin A has a potent anti-adipogenic effect and elucidated the molecular modulation underlying vitisin A activity in adipogenesis.

Materials and methods

Cell culture and reagents. 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. Insulin, 1-methyl-3-isobutyl xanthine (IBMX), and dexamethasone were purchased from Sigma (St. Louis, MO). Rosiglitazone was from Alexis

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(Bingham, Nottingham, UK), and Trizol Reagent from Invitrogen (Carlsbad, CA). Antibodies directed against PPARγ, C/EBPα, phospho-ERK (Thr202/Tyr204), and phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies specific for cyclin A, B, E, and CDK2, actin, p21 and phospho-Rb (Ser780) were from Santa Cruz Biotechnology (Santa Cruz, CA). All plasmids used in this study were the kind gifts of Professor Jae-Bum Kim (Seoul National University, Seoul, Korea). Vitisin A and other 17 stilbenes (listed in Table 1) were purified by conventional methods using sequential column chromatography in Korea Research Institute of Chemical Technology. Chemical structures were confirmed by direct comparison of their physical and spectral properties (^{1}H NMR and ^{13}C NMR).

Adipocyte differentiation and Oil Red O staining. Two days after confluence, designated day 0 (D0), 3T3-L1 cells were treated for 2 days with a differentiation medium (DM) containing 10 μg/ml insulin, 0.5 μM dexamethasone, and 0.5 mM IBMX. The medium was then changed to DMEM containing insulin and the cells continued to differentiate until day 8 (D8). Test compounds were administered during the adipogenic process. For the Oil Red O staining, on D8 the accumulated TG in the cells was stained with Oil Red O dye, as described by Kim et al. [12]. Briefly, cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS. Adipocytes were stained for 1 h with Oil Red O, which stained the fat droplets red. The stained lipid droplets were dissolved in isopropanol and quantified by measuring the absorbance at 510 nm.

Western blot analysis. Cells were washed with ice-cold PBS and lysed with lysis buffer (50 mM Tris–HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, and 0.2% protease inhibitor cocktail, pH 7.2). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane. Bands were detected by enhanced chemiluminescence (ECL) reagents (Amersham, Piscataway, NJ).

Transfection and reporter gene assay. COS-7 cells in 24-well plates were transiently transfected with 1 μg of total DNA (expression plasmids for 300 ng PPARγ, 300 ng RXRα; 300 ng luciferase reporter vector containing a PPAR-response element (PPRE), and 100 ng β-galactosidase), using SuperFect Transfection Reagent (Qiagen, Valencia, CA). Eighteen hours after transfection, the cells were treated with rosiglitazone in the absence or in the presence of vitisin A, and incubated for another 24 h. The cells were then used for a luciferase reporter gene assay with Luciferase Assay System (Promega, Madison, WI), and then normalized to β-galactosidase activity. Rosiglitazone 5 μM was used as a PPARγ activator. Transfection experiments were performed in triplicate and repeated independently at least three times.

Proliferation assay. Two days after confluence, 3T3-L1 cells were treated with DM containing vitisin A. At the indicated time points, 50 μg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) was added and incubated for 2 h. Treatment medium was removed and the observance was measured at 570 nm in a microplate reader (VersaMax, Molecular Device, Sunnyvale, CA).

Cytotoxicity assay. 3T3-L1 preadipocytes were grown in 48-well plate and incubated with either vehicle or vitisin A. At 24 h, released lactate dehydrogenase (LDH) into culture supernatant was measured by colorimetric cytotoxicity assay (CytoTox 96 nonradioactive cytotoxicity assay, Promega), as recommended by the manufacturer.

Flow cytometric analysis of cell cycle. Postconfluent 3T3-L1 preadipocytes were treated with DM in the presence or in the absence of vitisin A for 24 h. Then, cells were harvested and fixed with 70% ethanol at 4 °C for 1 h. After removing of ethanol, cells were stained with propidium iodide (Sigma) solution containing RNase (20 μg/ml) (Sigma) for 30 min. Fluorescence-activated cell sorting (FACS) analysis was performed on Becton–Dickinson FACScantoII instrument and data analysis with FACSDiva software (Becton–Dickinson, San Jose, CA).

Statistical analysis. All experiments were performed in triplicate and data are presented as means ± SD. Differences between means were assessed by one-way analysis of variance. For all analyses, the accepted level of significance was $P < 0.05$. Statistical analyses were conducted using SPSS 9.0 (SPSS Inc., Chicago, IL).

Table 1
The effects of 18 stilbene compounds on adipocyte differentiation

Compounds	IC_{50}[a] (μM)
Resveratrol	38.4
Stilbesterol	34.3
Ampelopsin A	25.4
Vitisin B	21.7
3,4′,5-Trimethoxy stilbene	7.7
Vitisin A	5.0
Piceatannol	Stimulation[b]

The following stilbenes had no effect on adipogenesis: 3,5-dihydroxy-4-methoxystilbene, 3,5-dihydroxy-4′-methoxystilbene, 3,5-dihydroxy-4-methylstilbene acetate, resveratrol-3-*O*-B-D-glucoside, resveratrol-3-*O*-Glu hexaacetate, resveratrol triacetate, rhaponticin, rhaponticin hexaacetate, rhapontigenin, rhapontigenin triacetate, and ε-viniferin.

[a] IC_{50} means the concentration required to inhibit 50% of adipocyte differentiation.

[b] Stimulates adipogenesis.

Results

Vitisin A inhibits 3T3-L1 adipocyte differentiation

The effect of 18 stilbene compounds on adipogenesis was evaluated, including resveratrol. As shown in Table 1, six compounds exhibited anti-adipogenic effects: stilbesterol, 3,5,4′-trimethoxystilbene, resveratrol, ampelopsin A, vitisin B, and vitisin A. Of these, vitisin A was the most effective with an IC_{50} value of 5.0 μM. Therefore, vitisin A was selected for further experiments. Oil Red O staining demonstrated that vitisin A inhibited 3T3-L1 adipocyte differentiation in a dose-dependent manner; at 10 μM of vitisin A, differentiation was almost suppressed (Fig. 1B and C).

Effect of vitisin A on the expression and transcriptional activity of PPARγ

The expressions of PPARγ and C/EBPα were examined, which are key transcription factors in adipogenesis, consequently used as adipogenic markers [6,13]. Coincident with the decrease in lipid content, PPARγ and C/EBPα expressions were also reduced by vitisin A treatment in a dose-dependent manner (Fig. 1D).

PPARγ transactivity was next studied to determine whether it was also altered by vitisin A. Although the inhibitory effect of vitisin A on PPARγ activity was statistically significant (Fig. 1E), the level of inhibition was weak and therefore not sufficient to explain the total reduction in the adipogenic phenotype (see Fig. 1B and C). Therefore, a search was conducted for a more specific target molecule of vitisin A than PPARγ.

Vitisin A affects the proliferation stage of the adipogenic process

The adipogenic process includes both preadipocyte proliferation and adipogenic differentiation [2,3]. Vitisin A was evaluated

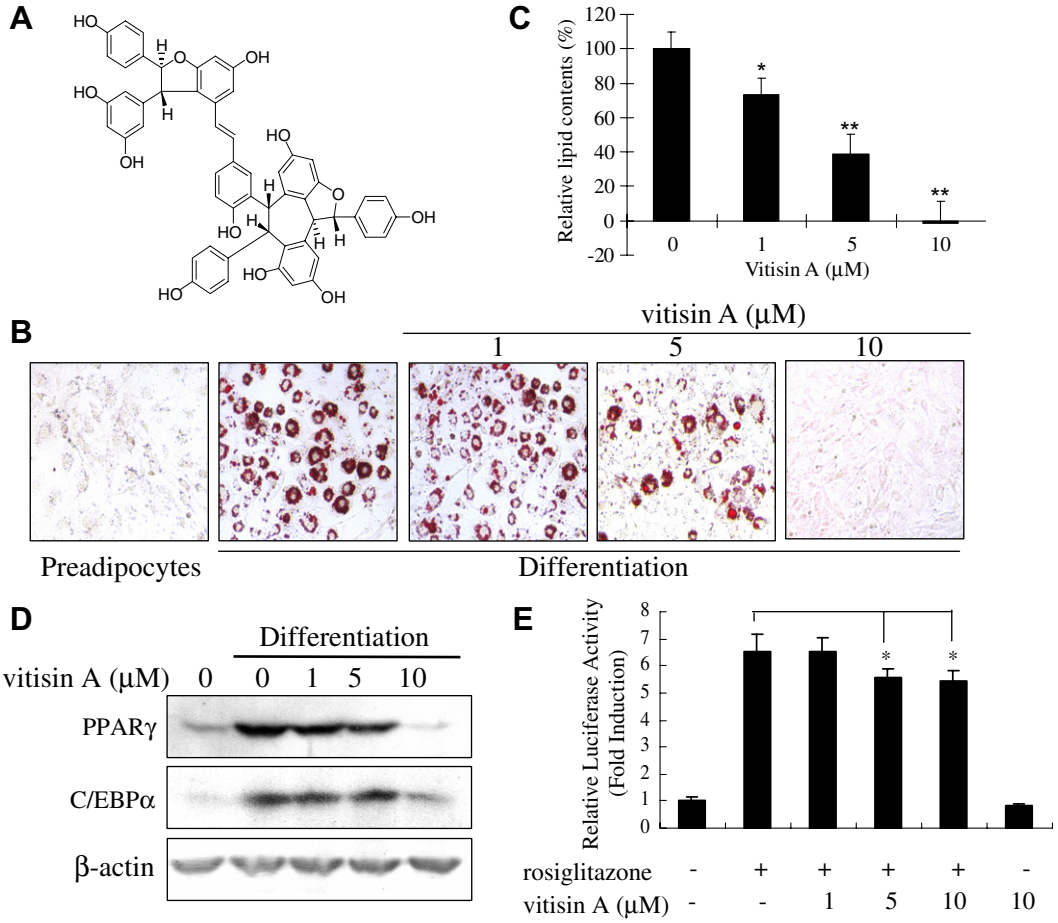

Fig. 1. Vitisin A inhibits 3T3-L1 preadipocyte differentiation. Postconfluent 3T3-L1 cells were differentiated in the absence or in the presence of vitisin A for 8 days. (A) Molecular structure of vitisin A. (B) The morphological changes associated with adipogenesis were photographed after Oil Red O staining. (C) The stained lipid content was quantified by measuring absorbance. The values given are relative to the values for the vitisin A-free control (deemed 100%). (D) At day 8, protein samples were subjected to Western blot analysis. (E) COS-7 cells were transfected as described under Materials and methods. Cells were treated with vehicle (DMSO), rosiglitazone, or vitisin A for 15 h. Transfection efficiencies were normalized to β-galactosidase activity. Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$.

to determine whether it affects the preadipocyte proliferation step. DM-induced mitosis was significantly inhibited by vitisin A treatment and the cell proliferation level was reduced nearly to that of normal growth-arrested cells (Fig. 2A). However, this inhibition was not due to a cytotoxic effect, because cytotoxicity appeared at greater than 50 μM (Fig. 2B).

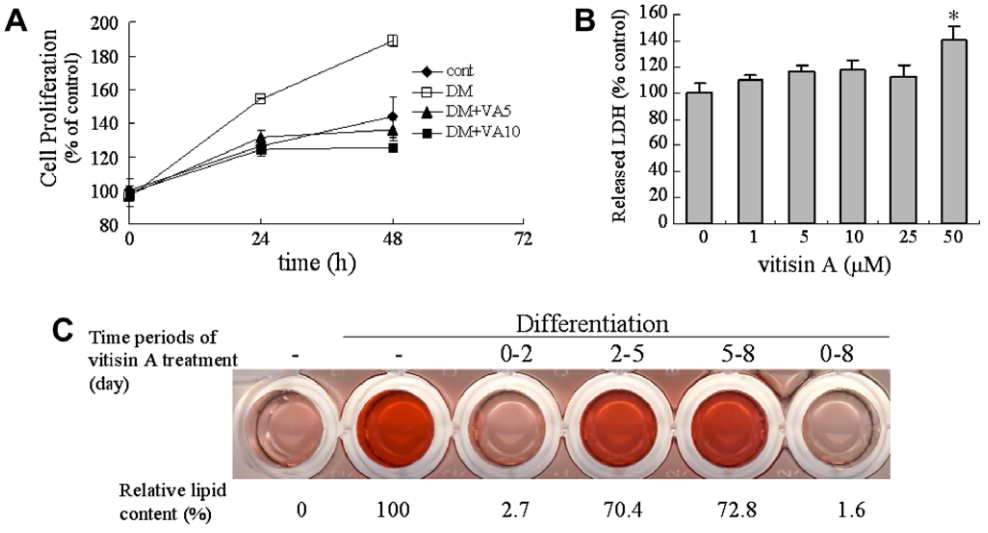

Fig. 2. Vitisin A inhibits DM-mediated cell proliferation. (A) Postconfluent 3T3-L1 cells were caused to differentiate with DM plus vitisin A. MTT assay was performed at 0, 24, and 48 h. VA:vitisin A. (B) Cytotoxicity assay. LDH content released into culture supernatant was measured. (C) 3T3-L1 cells were under DM for 8 days and vitisin A was added during the indicated time periods. On D8, accumulated lipid was stained and measured. Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$.

To confirm that vitisin A inhibits adipogenesis through suppression of preadipocyte proliferation, vitisin A was administered at different time periods. Mitosis of preadipocytes has been reported to actively occur within the first 2 days after adipogenic induction [2]. As expected, the inhibitory effect of vitisin A was outstanding during the first 2 days of treatment (Fig. 2C). Its effect during this time period (D0-2) was nearly equivalent to that over the entire 8 days of treatment. The administration of vitisin A after D2 (D2-5 and D5-8) resulted in a relatively low inhibition level; accumulated lipid contents in these treatments were 70.4% and 72.8% of the vitisin A-free control, respectively.

Vitisin A blocks cell cycle progression in 3T3-L1 cells

To prove the effect of vitisin A on cell mitosis after adipogenic induction, the cell cycle was analyzed by FACS. As has been previously reported [14], DM initiated cell cycle progression. However, vitisin A co-treatment blocked this cell cycle, especially at the G1/S transition and the DNA histogram type of vitisin A-treated cells was nearly the same as normal growth-arrested preadipocytes (Fig. 3A).

Reconfirmation of the vitisin A-dependent G1 arrest was attempted through analysis of cell cycle proteins using Western blotting. DM increased cyclin A and B expression over time, but these increases were significantly repressed by vitisin A in a dose-dependent manner (Fig. 3B and C). CDK2 expression was delayed by vitisin A, while cyclin E was not affected. Based on these results, vitisin A treatment decreased the level of proteins active during S and G2 phases of the cell cycle. This result was in accordance with the data obtained by FACS indicating G1 arrest induced by vitisin A.

Effect of vitisin A on several signaling molecules affecting the mitotic pathway

To elucidate the signaling pathway through which vitisin A affects preadipocyte proliferation, the involvement of extracellular signal-regulated kinases (ERK) or Akt pathways was first examined, because these signaling pathways have been reported to be related to preadipocyte proliferation [3,13]. However, in case of vitisin A, neither ERK nor Akt phosphorylation was affected with short-term treatment (Fig. 4A) or with long-term treatment (data not shown). On the other hand, the level of p21, a CDK inhibitor, was decreased during differentiation, but this decrease was restored by vitisin A co-treatment (Fig. 4B). Also, retinoblastoma protein (Rb) was phosphorylated during differentiation and vitisin A inhibited this phosphorylation. Based on these results, p21 and Rb seem to be involved in anti-mitotic regulation by vitisin A.

Discussion

Anti-obesity strategies are classified into four categories: reducing food intake, blocking nutrient absorption, increasing thermogenesis, and modulating fat or protein metabolism or storage [15]. Blocking of adipocytes differentiation is one of the anti-obesity strategies falling under the category of modulating fat storage. Utilization of anti-adipogenic compounds from natural sources could be helpful in the prevention of obesity, reducing side effects. Resveratrol, the most well-known stilbene, has gained interest due to its health-promoting activity, especially concerning adiposity or obesity. Resveratrol inhibits adipogenesis and stimulates breakdown of accumulated triglyceride in adipocytes [16]. It also improves health and survival in mice given a high fat diet [17].

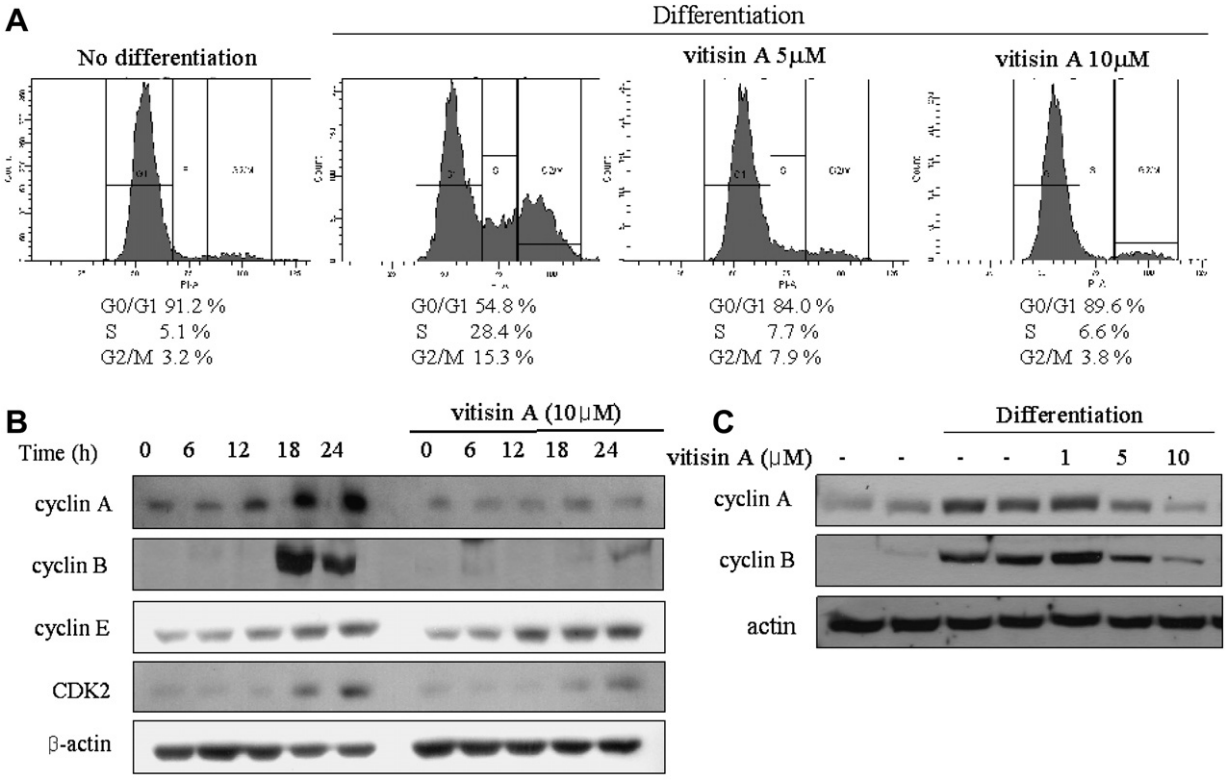

Fig. 3. Vitisin A causes G1 arrest in differentiation-induced cells. (A) 3T3-L1 cells were exposed to DM and/or vitisin A for 24 h. Cells were stained with PI solution and analyzed by FACS. (B) Postconfluent 3T3-L1 cells were differentiated in the presence or in the absence of 10 μM vitisin A. Proteins were harvested at indicated times. (C) Cyclin A and B expressions were decreased by vitisin A in a dose-dependent manner. Vitisin A was administered for 24 h.

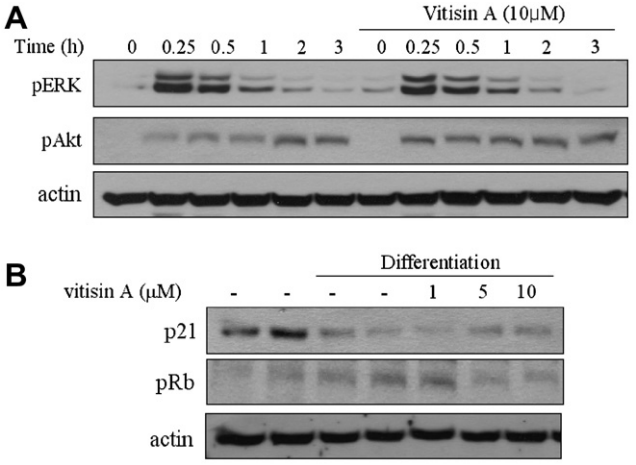

Fig. 4. Effect of vitisin A on several signal molecules involved in mitotic regulation. (A) Vitisin A was administered at 10 μM with DM. At indicated times, proteins were analyzed by Western blotting. (B) Vitisin A was administered to 3T3-L1 cells with DM for 24 h and then proteins were analyzed.

The present study demonstrated that vitisin A, a resveratrol tetramer, is more effective than resveratrol in the inhibition of adipogenesis. Its IC_{50} value (5 μM) is approximately eight times lower than that of resveratrol (38.4 μM). In addition, vitisin A demonstrated the most potent anti-adipogenic activity of 18 stilbene compounds studied. A representative adipogenic marker, PPARγ expression, was suppressed by vitisin A treatment. Meanwhile, PPARγ transcriptional activity was slightly reduced. Even at 10 μM of vitisin A treatment, PPARγ activity was attenuated a little, while adipogenesis was nearly blocked (compare Fig. 1E with B–D). Therefore, it was considered likely that another target molecule of vitisin A might exist upstream of PPARγ, because PPARγ expression was already reduced.

For differentiation from preadipocytes into mature adipocytes, proliferation of preadipocytes is a prerequisite. DM initiates this mitosis and adipogenic differentiation occurs after proliferation [2,3]. PPARγ is involved in the differentiation step [5,6], and therefore whether the inhibitory effect of vitisin A arises out of the preadipocyte proliferation step was subsequently examined. As has been previously reported, DM-induced cell proliferation is completed within the first 2 days, followed by the adipogenic differentiation process [14]. Vitisin A treatment during D0-2 almost entirely inhibited preadipocyte proliferation and consequent differentiation (Fig. 2A and C). Treatment during D2-5 and D5-8 with vitisin A also showed an anti-adipogenic effect, although the inhibition levels were relatively low. This inhibition might be derived from reduced PPARγ activity (Fig. 1E), because PPARγ is active during these periods [5,6].

FACS data on cell cycle analysis supported the effect of vitisin A on preadipocyte proliferation. Consistent with Tang et al. [14], DM caused the growth-arrested preadipocytes to initiate cell cycle progression, but vitisin A blocked the cell cycle at the G1/S transition (Fig. 3A). As can be seen in the DNA histogram, the sub-G1 population did not appear at all. Therefore, vitisin A does not induce apoptosis of adipocytes or preadipocytes. Cell cycle protein analysis also supported G1 arrest by vitisin A (Fig. 3B). At 0 h, 3T3-L1 cells were in the G1 phase, because all protein markers were expressed at a low level. However, expressions of the markers cyclin A, B, E, and CDK2 increased with time after induction. Vitisin A co-treatment significantly reduced cyclin A and B levels, and this prominent difference of expression between vitisin A-treated and vitisin A-free cells occurred at 12–18 h. According to a previous report [14], the G1-S check point and initiation of the S phase occurs in this time period. Also, cyclin A and B are active in the S and G2 phases of the cell cycle. Taken together, these results indicate that vitisin A blocks the cell cycle at the G1/S phase transition and causes preadipocytes to remain at the G1 phase, even though the adipogenic signal stimulates the cells.

The study next examined which signal pathway vitisin A uses to regulate the mitotic cascade. ERK is a representative cell survival and proliferation signal transducer. EGCG is one of the green tea cathechins, downregulates preadipocyte proliferation through the ERK pathway during adipogenesis [3]. On the other hand, Akt also gives a cell survival and proliferation signal and *Hibiscus* extract modulated the Akt signaling pathway during adipogenesis [13]. Although that experiment did not distinguish mitosis from adipogenesis in general, blocking Akt with PI3-kinase inhibitors suppressed adipogenesis [13]. In the case of vitisin A, the ERK and Akt signaling pathways were examined to determine whether they are also involved in vitisin A-dependent anti-mitosis. However, neither one was affected by either with long-term or short-term treatment with vitisin A (Fig. 4A). Instead, p21 and Rb were modulated by vitisin A treatment. Once the cell cycle progression was initiated, CDK inhibitor p21 expression was decreased; however, this reduction was restored by vitisin A treatment. Rb is an important regulator of the G1 to S phase transition in the cell cycle and represses gene transcription required for this transition by interacting with E2F transcription factors [18]. Rb is regulated by CDK phosphorylation, and the Rb phosphorylation state continues from the late G1 phase of the cell cycle until mitosis [19]. The present data showed that onset of differentiation initiated Rb phosphorylation; however, vitisin A reduced this phosphorylation. Based on these results, vitisin A seems to downregulate the p21 level and CDK activity in a sequential manner. Reduced Rb phosphorylation due to inactive CDK suppresses the cell cycle progression at the G1/S checkpoint by interacting with E2F and repressing transcription. However, additional studies are needed to confirm whether the activities of E2F binding/transcription and CDK are modulated directly by vitisin A-dependent p21 expression.

PPARγ has been used not only as a representative adipogenic marker but also an important target molecule for inhibition of adipogenesis. However, for many of the anti-adipogenic compounds, it has been reported that their inhibitory effects arise from anti-mitotic activity, rather than PPARγ activity modulation, including berberine [20], genistein [21], AICAR [22], and EGCG [23]. Thus, inhibition of adipogenesis through modulation of preadipocyte proliferation could be applicable as an anti-obesity target marker.

In conclusion, vitisin A, a plant-derived natural compound, inhibits adipocyte differentiation through p21- and Rb-dependent cell cycle arrest and consequent suppression of preadipocyte proliferation.

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