Research Article

Rubi Fructus (*Rubus coreanus*) Inhibits Differentiation to Adipocytes in 3T3-L1 Cells

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Rubi Fructus (RF) is known to exert several pharmacological effects including antitumor, antioxidant, and anti-inflammatory activities. However, its antiobesity effect has not been reported yet. This study was focused on the antidifferentiation effect of RF extract on 3T3-L1 preadipocytes. When 3T3-L1 preadipocytes were differentiating into adipocytes, 10–100 μg/mL of RF was added. Next, the lipid contents were quantified by Oil Red O staining. RF significantly reduced lipid accumulation and downregulated the expression of peroxisome proliferator-activated receptor γ (PPARγ), CCAAT-enhancer-binding proteins α (C/EBPα), adipocyte fatty acid-binding protein 2 (aP2), resistin, and adiponectin in ways that were concentration dependent. Moreover, RF markedly upregulated liver kinase B1 and AMP-activated protein kinase (AMPK). Interestingly, pretreatment with AMPKα siRNA and RF downregulated the expression of PPARγ and C/EBPα protein as well as the adipocyte differentiation. Our study shows that RF is capable of inhibiting the differentiation of 3T3-L1 adipocytes through the modulation of PPARγ, C/EBPα, and AMPK, suggesting that it has a potential for therapeutic application in the treatment or prevention of obesity.

1. Introduction

Obesity is associated with many diseases inducing diabetes, dyslipidemia, and atherosclerosis, which are risk factors for metabolic syndrome [1]. Obesity is a condition in which excess body fat has accumulated due to lipids changing into adipocytes and an increase in the number of differentiated mature cells which are regulated by genetic and environmental factors such as nutrients [2–4]. Understanding the mechanism through which a particular nutrient affects the differentiation to adipocytes would help to prevent the initiation and progression of obesity. The 3T3-L1 cell line is one of the best-characterized and reliable models for studying the conversion of preadipocytes into adipocytes. Adipocyte differentiation is a complex process involving coordinated expression of specific genes and proteins associated with each stage of adipogenesis [5, 6].

Peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα) have been known to play a key role in the regulation of adipogenesis and in the modulation of fat cell function in adipose tissue. PPARγ, a member of the PPAR subfamily of nuclear hormone receptors, was identified as a component of a differentiation-dependent regulatory factor and a far-cell-specific enhancer of the adipocyte fatty acid-binding protein (aP2) gene [7, 8]. Another major protein in obesity regulation, C/EBPα, a member of a large family of leucine zipper transcription factors, plays an important role in induction of terminal adipocyte differentiation [9, 10]. PPARγ and C/EBPα are not
expressed in preadipocytes but are activated during adipocyte differentiation. PPARγ and C/EBPα are expressed prior to the expression of most adipocyte genes and regulate the expression of genes involved in creating and maintaining adipocytes, including aP2 and resistin [11, 12].

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that is widely expressed in eukaryotes. AMPK, composed of α, β, and γ subunits, is a key player in energy homeostasis. The α subunit is the catalytic subunit, and its activation via the phosphorylation of the threonine residue T172 by the upstream liver kinase B1 (LKB1) is crucial for AMPK activation under ATP-depleted conditions [13]. When the intracellular AMP/ATP ratio increases because of the metabolic stress, AMPK is phosphorylated. Subsequently, downstream target molecules are activated, promoting catabolism. When the intracellular AMP/ATP ratio decreases, AMPK increases the anabolism. AMPK is associated with adipocyte differentiation via AMPK activation in 3T3-L1 adipocytes [14, 15]. In addition, AMPK inhibits the accumulation of fat by modulating downstream substrate acetyl-CoA carboxylase (ACC) [16].

Rubi Fructus (RF), the fruit of Rubus coreanus Miquel (Rosaceae), is a type of red raspberry from southern Korea. The dried fruit are used in traditional herbal medicine for the treatment of impotence, spermatorrhea, enuresis, and asthma [17]. RF includes functional constituents that include flavonoids, anthocyanin, polyphenols, nigaichigoside F1, 23-hydroxytormentic acid, and gallic acid [18, 19]. It has been found that these constituents show anticarcinogenic, antiinflammatory effects [20, 21]. Even though numerous biological activities of RF have been reported, there is limited evidence for its antiobesity effect. In this study, we evaluated the inhibitory effect of RF and investigated how it acts to reduce differentiation to adipocytes in 3T3-L1 mouse fibroblasts.

2. Materials and Methods

2.1. Preparation of RF. Dried and ground powder of RF, the fruit of Rubus coreanus Miquel (Rosaceae), was kindly provided by Kyung Hee Oriental Hospital (Seoul, Republic of Korea). To prepare the aqueous extract, the water-soluble components of the RF powder were extracted with water (100 g/L of water) by heating at 100 °C for 3 h. The boiled solution was filtered through Whatman filter paper, and the filtrates were lyophilized. The resulting powder was used as the crude total extract of the fruit. Total extracts were dissolved in water for cell treatment. The crude extract (10 g) was subsequently partitioned between ethyl acetate (EtOAc) and distilled water. The EtOAc fraction was concentrated under reduced pressure in a rotary evaporator and lyophilized. The EtOAc fractions were dissolved with DMSO, filtered using sterilized syringe filter through a 0.22 μm membrane pore, and stored at −20 °C before use.

2.2 Reagents. Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin, fetal calf serum (FCS), and fetal bovine serum (FBS) were from Gibco BRL (Grand Island, NY, USA). Insulin, 3-isobutylmethylxanthine (IBMX), and dexamethasone (DEX) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-C/EBPα, anti-resistin, and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PPARγ, anti-aP2, anti-adiponectin, anti-phospho LKB1, anti-phospho AMPKα anti-AMPKγ, and anti-ACC antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.3. Cell Culture and Adipocyte Differentiation. 3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in DMEM plus 10% calf serum and plated for final differentiation in DMEM plus 10% FBS with 100 units/mL of penicillin-streptomycin solution at 37 °C, in 5% CO₂, at 95% humidity until confluence. Two days after confluence (Day 0), the cells were stimulated to differentiate with differentiation inducers (1 μM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine, and 1 μg/mL insulin, MDI) that were added to DMEM containing 10% FBS for two days (Day 2). Preadipocytes were then cultured in DMEM, 10% FBS supplemented with 1 μg/mL insulin for another two days (Day 4), followed by culturing with 10% FBS/DMEM medium for an additional two days (Day 6), at which time more than 90% of cells were mature adipocytes with accumulated fat droplets. On Day 2, RF was prepared in a differentiation medium at concentrations of 10 μg/mL, 50 μg/mL, and 100 μg/mL.

2.4. Cell Cytotoxicity Assay. Cell viability was measured with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega Corporation, Madison, USA) according to the manufacturer’s instruction. Briefly, The cells (5 × 10⁴ per 96 well) were incubated at 37 °C in 5% CO₂ and 95% air with different concentrations of RF. After 48 h for 3T3-L1 preadipocytes, 20 μL of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium, inner salt] solution was added to each well, incubated for 4 h, and absorbance at 490 nm was measured using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) to determine the formazan concentration, which is proportional to the number of live cells.

2.5. Oil Red O Staining. Intracellular lipid accumulation was measured using Oil Red O. The Oil Red O working solution was prepared as described by Ramirez-Zacarias et al. [22]. The 3T3-L1 cells were washed twice with phosphate-buffered saline (PBS) and were then fixed in 10% formaldehyde in PBS for 1 h. After washing with 60% isopropanol, the cells were stained with Oil Red O solution for 30 min at room temperature. The cells were washed with water four times to remove the unbound dye. The stained cells were observed with an Olympus IX71 Research Inverted Phase microscope (Olympus Co., Tokyo, Japan). Following the microscopic observation, 100% isopropanol was added as an extraction solution to extract the excess staining dye from the cells. The absorbance of the extracted dye was measured spectrophotometrically at 500 nm in a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).
2.6. RNA Isolation and Real-Time RT-PCR. Total RNA was extracted using a GeneAll RiboEx Total RNA extraction kit (GeneAll Biotechnology, Seoul, Republic of Korea) and QIAzol lysis reagent (QIAZEN sciences, Maryland, USA). RNA (2 μg) was used as a template for first-strand cDNA synthesis performed using a Power cDNA Synthesis Kit (INTRON Biotechnology, Seoul, Republic of Korea) according to the manufacturer's instructions. Newly synthesized cDNA from 3T3-L1 control cells and RF-treated cells was amplified using specific primers and the Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR products were measured with a StepOnePlus Real-Time RT-PCR System, and the relative gene expression was calculated based on the comparative CT method using a StepOne software (Bio-Rad Laboratories, Hercules, CA, USA). All experiments were repeated at least three times. Representative Western blots are shown along with the graphs of the quantitative data. The chemiluminescent intensities of protein signals were quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Western Blot Analysis. T3-L1 cells were harvested and washed with PBS and then collected by centrifugation at 13,000 rpm for 1 min at 4°C. To obtain the cellular lysate, cells were lysed on ice for 30 min in RIPA buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM DTT, and 1 mM PMSF), which contained a mixture of protease inhibitors (Sigma, Mannheim, Germany). Insoluble materials were removed by centrifugation at 13,000 rpm for 15 min at 4°C. A total of 20 μg of the supernatants were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 10 mM Tris, 150 mM NaCl, and 0.05% Tween-20 (TBST) (pH 7.6) containing 5% skim milk-TBST at room temperature for 1h. Protein signals were developed using the ECL Western Blotting Detection Reagent (Amersham Bioscience, Piscataway, NJ, USA). Values with *P < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Effects of RF on Cytotoxicity and Inhibition of Adipogenesis in 3T3-L1 Adipocytes. To determine the cytotoxicity of RF, 3T3-L1 cells were treated with various concentrations (10–500 μg/mL) of RF, and the cell viability was measured by the MTS assay. As shown in Figure 1(a), treatment with 10–100 μg/mL of RF did not cause significant cytotoxic effects.
3.2. Comparative Effects of RF Extraction Fraction on Inhibition of Adipogenesis in 3T3-L1 Adipocytes. To determine the comparative effects of RF extraction fraction, total water-soluble extract was sequentially extracted with ethyl acetate (EtOAC fraction, RFE) and water (water fraction, RFW). The 3T3-L1 cells were treated with three extracts together with MDI, and then the extent of adipocyte differentiation was determined by the Oil Red O staining method. As shown in Figures 2(a) and 2(b), among the three extracts, total water-soluble extract of RF (RF) was most effective in inhibiting adipocyte differentiation indicating that antiadipogenic activities might have been enriched RF. Troglitazone, one of the antidiabetic drugs and a ligand for PPARγ, was used as a positive control, and 2-chloro-5-nitro-N-phenylbenzamide GW9662, an antagonist for PPARγ, was used as a negative control.

3.3. Effect of RF on the Expression of PPARγ and C/EBPα in 3T3-L1 Adipocytes. To investigate whether RF suppresses adipogenesis through a PPARγ pathway, gene expressions of PPARγ and C/EBPα were evaluated by quantitative real-time RT-PCR and Western blot analysis, respectively, after the treatment of fully differentiated cells with 10–100 μg/mL of RF. We observed that the expressions of PPARγ and C/EBPα were strongly inhibited by RF at the mRNA level (Figures 3(a) and 3(b)). We also demonstrated that RF treatment resulted in a dose-dependent suppression of PPARγ and C/EBPα at the protein level. PPARγ and C/EBPα protein levels were reduced up to 68% by treatment with 100 μg/mL of RF (Figures 3(c) and 3(d)).

3.4. Effect of RF on Expression of aP2, Resistin, and Adiponectin in 3T3-L1 Adiposity. We next examined the effects of RF on the expression of adipogenic genes such as aP2, resistin, and adiponectin in 3T3-L1 cells. Fully differentiated cells were treated with 50 or 100 μg/mL of RF, followed by extraction of total RNA for analysis using quantitative real-time PCR. RF treatment with 0.05 mg/mL or 0.1 mg/mL significantly decreased the expression of aP2, resistin, and adiponectin (Figures 4(a), 4(b), and 4(c)). In particular, the resistin mRNA level was reduced up to 53% by treatment with...
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Figure 2: Effect of RF fraction on lipid accumulation of 3T3-L1 adipocyte differentiation. Postconfluent 3T3-L1 cells were differentiated in the absence or presence of RF, RF ethyl acetate fraction (RFE), and RF water fraction (RFW) for 6 days. (a) Lipid droplets were measured by Oil Red O staining. (b) Lipid content was quantified by measuring absorbance. Troglitazone was used as positive control, and GW9662 was used as negative control. Data are expressed as the means ± S.D. where $P < 0.05$ was considered a statistically significant difference from the differentiated control.

3.5. The Effect of RF and AMPK siRNA Pretreatment on the Expression and Phosphorylation of Proteins Related to Adipogenesis. LKB1, which is an upstream kinase of AMPK, activates AMPK protein in adipose tissue [23]. To investigate whether AMPK, a key player in energy homeostasis [7], is activated by RF during 3T3-L1 differentiation, the protein levels of p-LKB1, p-AMPKα, and ACC were analyzed. When compared with the control group, LKB1 and AMPKα phosphorylation was increased by treatment with RF. The observed increase in the phosphorylation of LKB1 by RF suggests that RF might upregulate AMPK activity via LKB1. However, the expression of ACC, a downstream target protein of AMPK, was significantly suppressed (Figures 5(a) and 5(b)). To further confirm the above results, adipocytes were pretreated with AMPKα siRNA and then treated with 100 μg/mL of RF, and the relative intracellular fat content of each group was determined. AMPKα siRNA pretreatment effectively decreased the relative intracellular fat content when compared with the control group suggesting that AMPKα siRNA can inhibit adipocyte differentiation with or without RF treatment (Figure 5(c)). Figure 5(d) shows that the AMPKα expression was decreased at the protein level after the siRNA treatment. In addition, both RF and AMPKα siRNA treatments decreased the expression of PPARγ and C/EBPα proteins.

4. Discussion

Obesity is caused by the accumulation of lipid through adipogenesis in adipose tissue, and adipogenesis is the process by which undifferentiated precursor cells differentiate into fat storage cells. Numerous studies have demonstrated that adipocyte differentiation and the amount of fat accumulation are associated with the occurrence and development of obesity. Therefore, the inhibition of adipocyte differentiation is one of the strategies for the treatment of obesity. Recent studies have attempted to investigate the beneficial effects of natural products on obesity. The present study demonstrates the novel effect of RF extract on the inhibition of adipocyte differentiation. Our results show that RF did not cause significant cytotoxic effects in 3T3-L1 cells and significantly inhibited lipid accumulation and adipocyte differentiation in a concentration-dependent manner. These results indicate that RF inhibited adipogenesis during adipocyte differentiation and may have potential antiobesity effects (Figure 1). We also demonstrated that treatment with total water-soluble extract of RF strongly inhibited adipogenesis during adipocyte differentiation relative to an ethyl acetate-soluble fraction of RF (RFE) or water fraction of RF (RFW) (Figure 2).

PPARγ and C/EBPα are known to play a role in fat cell function and adipocyte differentiation of preadipocytes [24]. PPARγ induces C/EBPα and also increases its own expression. Similarly, C/EBPα induces PPARγ expression as
well as its own expression. These cooperative functions help in maintaining high levels of PPARγ and C/EBPα, and then PPARγ stimulates adipocyte differentiation. Rosen et al. [25] reported that C/EBPα can support adipocyte-specific gene expression in the presence of PPARγ at the level of cell morphology and lipid accumulation. In this study, we investigated whether RF can inhibit adipocyte differentiation through the suppression of related transcription factors such as PPARγ- and C/EBPα. As a result, RF treatment resulted in reduced expression of PPARγ and C/EBPα at both the mRNA and protein levels in 3T3-L1 cells (Figure 3). These results indicate that RF suppresses adipocyte differentiation through a PPARγ and C/EBPα-mediated adipogenesis mechanism. We also observed that 3T3-L1 cells treated with RF showed a decreased protein expression as well as mRNA expression of several adipogenesis-related genes including aP2, resistin, and adiponectin, in a dose-dependent manner (Figure 4). A member of the cytoplasmic fatty acid-binding protein family, aP2, was detected in adipose tissue, and its expression was highly regulated during the differentiation of adipocytes [26]. PPARγ binding is required for the function of the fat-selective enhancer for the aP2 gene, in cultured fat cells [24]. Resistin, an adipocyte-secreted molecule, serves as a critical link between obesity and insulin resistance and plays a role in the regulation of glucose homeostasis and hepatic glucose production [27, 28]. Acute administration of recombinant resistin to rats results in impaired glucose tolerance and hepatic insulin resistance [28]. Previous studies have demonstrated that compounds with antiobesity activity inhibit adipocyte differentiation in 3T3-L1 cells through the downregulation of PPARγ, C/EBPα, aP2, and resistin [29, 30]. Adiponectin is an adipocyte-derived hormone that plays a role in insulin sensitivity and energy homeostasis [31]. Previous studies have demonstrated that PPARγ agonists can induce an increase in adiponectin levels in 3T3-L1 adipocytes, and that this effect is associated with adipocyte differentiation via PPAR response element [32, 33].

AMPK, a central sensor of cellular energy, is a eukaryotic heterotrimeric serine/threonine kinase, and it has emerged as a therapeutic target for metabolic disorders including obesity. The activation of AMPK is essential for the inhibition of 3T3-L1 adipocyte lipogenesis by phytochemicals [34]. To
Figure 4: Effect of RF on the expression of aP2, resistin, and adiponectin in 3T3-L1 cells. Postconfluent 3T3-L1 cells were differentiated in the absence or presence of RF (0, 10, 50, and 100 μg/mL) for 6 days. (a) aP2, (b) resistin, and (c) adiponectin mRNA expression were evaluated by the quantitative real-time PCR. (d) aP2, resistin, and adiponectin protein levels were analyzed by Western blot analysis. GAPDH was used as internal controls. Data are expressed as means ± S.D. where P < 0.05 was considered a statistically significant difference from the differentiated control.

5. Conclusion

In conclusion, the objective of this study was to elucidate the effect of RF on adipogenesis in 3T3-L1 cells. This study demonstrated that the extract of RF inhibited adipocyte differentiation of 3T3L-1 and fat accumulation. The antiadipogenic mechanism of RF involves the downregulation of the adipogenic transcription factors, PPARγ and C/EBPα, which are related to the expression of aP2, resistin, and adiponectin. In addition, we observed that RF increases
AMPK phosphorylation, which plays major roles in the expression of PPARγ and C/EBPα. These findings suggest that RF might have a therapeutic effect in the prevention of adipogenesis-related obesity and may be a potential natural drug candidate for the treatment of obesity.

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