Anti-obesity effect of resveratrol-amplified grape skin extracts on 3T3-L1 adipocytes differentiation

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Abstract
Resveratrol (3,4,5-trihydroxy-trans-stilbene), a phytoalexin found in grape skin, grape products, and peanuts as well as red wine, has been reported to have various biological and pharmacological properties. The purpose of this study was to investigate the anti-obesity effect of resveratrol-amplified grape skin extracts on adipocytes. The anti-obesity effects of grape skin extracts were investigated by measuring proliferation and differentiation in 3T3-L1 cells. The effect of grape skin ethanol extracts on cell proliferation was detected by the MTS assay. The morphologic changes and degree of adipogenesis of preadipocyte 3T3-L1 cells were measured by Oil Red-O staining assay. Treatment with extracts of resveratrol-amplified grape skin decreased lipid accumulation and glycerol-3-phosphate dehydrogenase activity without affecting 3T3-L1 cell viability. Grape skin extract treatment resulted in significantly attenuated expression of key adipogenic transcription factors, including peroxisome proliferator-activated receptor, CCAAT/enhancer-binding proteins, and their target genes (FAS, aP2, SCD-1, and LPL). These results indicate that resveratrol-amplified grape skin extracts may be useful for preventing obesity by regulating lipid metabolism.

Key Words: Grape skin extracts, resveratrol, 3T3-L1, adipocyte differentiation

Introduction
Resveratrol (3,4,5-trihydroxy-stilbene) is a naturally occurring polyphenol found in different plant species [1]. Considerable amounts of resveratrol are found in grapes, berries, and peanuts. Besides natural sources, this compound has become recently available in tablets and is recommended as a dietary supplement. In the past few years, interest in resveratrol has increased substantially, and its broad biological activity at the cellular level has been demonstrated [2]. Supplementing mice fed a high fat diet with resveratrol increases mitochondrial content/activity in skeletal muscle brown adipose tissue and the liver to protect against developing diet-induced obesity and improving metabolic disturbances [3]. Moreover, the cardioprotective, anti-cancer, anti-inflammatory, and antioxidant properties of resveratrol are well characterized [4-7]. Recent data derived from animal studies have opened a new, promising perspective for the potential use of resveratrol to prevent serious metabolic disorders such as obesity and diabetes [1,8].

The prevalence of obesity is increasing worldwide [9]. According to the World Health Organization, obesity is a global epidemic, due to the parallel rise of related morbidity and risk factors. Obesity, which is characterized by excess fat, results from interactions between genetic and environmental factors. It is well known that a fat-enriched diet leads to the accumulation of adipose tissue and to the development of metabolic alterations associated with weight gain, particularly in genetically predisposed individuals [10]. Obesity is linked to a variety of metabolic disorders such as insulin resistance and atherosclerosis [11]. Environment, lifestyle, and genetic susceptibility probably contribute to the increased risk of obesity.

Recent data indicate that resveratrol plays a crucial role in the cardiovascular protection provided by grapes and wines [12]. Grapes contain various nutritive elements such as vitamins, minerals, carbohydrates, and fiber. Polyphenols are the most important phytochemicals in grapes, because they possess many biological activities and health-promoting benefits [13]. Recent studies have revealed that resveratrol and proanthocyanidins are the important polyphenolic antioxidants present in grapes. Proanthocyanidins are found in both the skin and seeds of grapes, but resveratrol is mainly found in grape skin [14].

Resveratrol content decreases due to direct sunlight exposure, but ultraviolet (UV) irradiation induces resveratrol biosynthesis in grapes [15]. Recently, Cho et al. [16] showed that resveratrol content in grapes treated with UV irradiation increases five times more than that in untreated grapes.
The present study was conducted to evaluate the antiobesity activity of resveratrol-amplified grape skin extracts in 3T3-L1 preadipocytes and to elucidate the mechanism underlying such an effect as exerted in visceral adipose tissue.

**Materials and Methods**

**Preparation of resveratrol-amplified grape skin extracts**

Grape (*Vitis labruscana* Bailey, Campbell Early) was purchased from Yeongcheon-si Gyeongsangbuk-do, Korea. The grapes were cleaned by sonication (45-50 kHz) for 3-5 min and passed through an amplifier (UV-irradiation, 0.5-1.0 mW/cm²) at 253.7 nm and then stored at 15-25°C for 3-6 days to enhance resveratrol content. Grape skin was separated from grape flesh and dried with lyophilizer (MCFD 5510, Eyela, Tokyo, Japan). A powder form of the grape skin was obtained by grinding the skins with a grinder. The grape skin powder was stored at -70°C until use.

Resveratrol-amplified grape skin powder was weighed (500 g) and extracted twice with ethanol/water (50/50 [v/v]) mixture and ethanol/water (80/20 [v/v]) mixture, respectively. The filtrates were combined and evaporated under a vacuum (Buchi, Berlin, Germany). The yields of the 50% and 80% ethanol extracts of resveratrol-amplified grape skin were 59.3% and 47.9% based on dry weight, respectively.

**Physiological activity of resveratrol-amplified grape skin extracts**

Total polyphenol content was determined using a colorimetric assay based on the method of Singleton and Rossi [17]. Total antioxidant capacity was measured according to the method of Re et al. [18] and expressed as a Trolox equivalent value. Resveratrol content was analyzed using high performance liquid chromatography (Shimadzu SCL-10Avp, Japan) with a UV-Vis detector at 320 nm.

**Cell culture differentiation**

Mouse embryo 3T3-L1 fibroblasts were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown at 37°C under a humidified 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum and 100 U/ml penicillin-streptomycin. Two days after confluence, preadipocytes of 3T3-L1 (designated as day 0) were cultured in differentiation medium (DM) containing 10% fetal bovine serum [FBS], 10 µg/mL insulin, 0.5 mM isobutylmethylxanthine, and 1 µM dexamethasone. After 2 days, the medium was switched to post DM containing 10% FBS and 10 µg/mL insulin for four days, and then changed to 10% FBS medium for an additional 2 days to induce differentiation.

**Cell viability assay**

Cells (5 × 10³/96 well) were incubated with resveratrol-amplified grape skin extracts at various concentrations (0, 30, 60, 120, 250, 500, and 1,000 µg/ml) for 24 and 48 h. The control cells were received the same amount of DMSO. Twenty µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution was added to each well. After incubation for 20 min, absorbance was measured at 490 nm using a microplate reader (Sensident scan, Labsystems, Helsinki, Finland).

**Oil Red-O staining**

Oil Red-O staining was performed on day 8. 3T3-L1 adipocyte cells were washed with phosphate buffered saline (PBS) and fixed with 10% formalin. After Oil Red-O stain, cells were photographed using a phase-contrast microscope (Olympus CKX41, Tokyo, Japan) in combination with a digital camera at 100 × magnification. The lipid droplets were dissolved in isopropanol and measured at 540 nm.

**Glycerol-3-phosphate dehydrogenase (GPDH) activity**

Resveratrol-amplified grape skin extract-treated 3T3-L1 adipocytes were harvested on day 8, washed with PBS, and collected using lysis buffer (25 mM Tris buffer containing 1 mM EDTA and 1 mM DTT (pH 7.5)). The harvested cells were sonicated and then centrifuged at 10,000 rpm for 5 min. The GPDH activity in supernatants was measured with assay kit (Takara Bio, Shiga, Japan).

**RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from 3T3-L1 cells using the

<table>
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<th>Gene symbol</th>
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<th>Primer sequence</th>
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