Downregulated Lipid Metabolism in Differentiated Murine Adipocytes by Procyanidins from Defatted Grape Seed Meal

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Recent reports have proposed possible anti-obesity mechanisms for antioxidants involving increased energy expenditure, pre-adipocyte differentiation and proliferation, decreased lipogenesis and increased lipolysis, and fat oxidation. The aim of this study was to examine and to confirm the anti-obesity effect of the oligomeric and polymeric proanthocyanidin fractions from defatted grape seeds. The lipid metabolism-related mRNA level in the mouse preadipocytes, 3T3-L1 cells, was determined to evaluate the anti-obesity effect of the phenolic fractions from a grape seed meal. Lipid accumulation was reduced by 19% of the control level by the procyanidin fraction originating from the grape seed meal.Emerging from the effect of the treatment on HSL and LPL mRNA expression, lipolytic enzyme activity was not involved in the anti-obesity effects of CPE and FPP from the defatted grape seed meal. We tested and confirmed in this study the effect of the biological activities of oligomeric and polymeric procyanidins from the defatted grape seed meal. It is suggested from the results of this brief study that further studies would be desirable to focus on the anti-obesity effect of the purified extracts of a defatted grape seed meal.

Key words: defatted grape seed meal; 3T3-L1; proanthocyanin; lipid metabolism

Proanthocyanidins, which are abundant from various natural sources such as apples,10 cranberries,11 jujube (Ziziphus jujuba) fruits,12 cocoa,5,7 are known to have beneficial effects on health: anti-tumoral,5,8,9 anti-inflammatory,10,11 neuroprotective,12 hypotensive or vasorelaxant,13-15 and cardioprotective.16 An antioxidative function is believed to be a common characteristic of proanthocyanidins which enables these chemopreventive effects. The antioxidation by proanthocyanidins is mediated by scavenging reactive oxygen species (ROS), including hydrogen peroxide (H$_2$O$_2$), the superoxide anion, and hydroxyl radicals.

Grape-originated proanthocyanidins have assumed importance, since grape-based products, primarily wine, are a significant dietary source of proanthocyanidins in respect of the amount and pattern of human consumption.17) Grape seeds are one of the major byproducts of wine and grape juice production. Grape seeds have recently been used for the production of seed oil which is a valuable food product and cosmetic ingredient. A secondary byproduct from the oil extraction process is the defatted grape seed meal, plausibly believed to be a rich source of proanthocyanidins, since the proanthocyanidin distribution in grapes is not restricted to the fruits but also in the leaves.18-20 peel,21 stem,22 and seeds.23-26 The defatted grape seed meal has been confirmed as a rich source of such monomeric phenolic compounds as catechin, epicatechin and epicatechin gallate, and of dimeric, oligomeric, and polymeric procyanidins which are known to have pharmacological and therapeutic potential.27 However, the biological activities of oligomeric and polymeric procyanidins from defatted grape seed meal are largely unknown.

Recent reports have proposed possible anti-obesity mechanisms for antioxidants involving increased energy expenditure, pre-adipocyte differentiation and proliferation, decreased lipogenesis and increased lipolysis, and fat oxidation.28 We have previously demonstrated antioxidative and cytoprotective effects in PC 12 cells.29

The aim of this present study was to examine and confirm the anti-obesity effect of the oligomeric and polymeric procyanidin fractions from defatted grape seed. The lipid metabolism-related mRNA level in the mouse preadipocytes, 3T3-L1 cells, was determined to evaluate the anti-obesity effect of the phenolic fractions from grape seed meal.

Materials and Methods

Sample preparation. Samples from defatted grape seed meal were prepared by the method previously described.30 Briefly, 100 g of defatted grape seed meal was obtained from the Campbell early grape variety, which is widely cultivated in Korea, and extracted with 75% (vol/vol) acetone and then by 80% (vol/vol) methanol. The combined supernatants were evaporated to remove the organic solvents, subjected to hexane extraction to eliminate the fatty materials, and then passed through a membrane filter (0.45 μm pore size). The aqueous polyphenolic solution was stored at −20°C prior to fractionation in C18 open columns or was lyophilized to obtain a brownish-yellow powder, referred to as the crude polyphenol extract (CPE). Fractionation was conducted by loading 50 mL of the aqueous polyphenolic solution (13.6 mg of dry matter/mL) into an open column (200 × 25 mm i.d.) packed with Cosmosil® 75C18-OPN (75 μm particle size; Nacalai Tesque, Kyoto, Japan). Elution began with...
100 mL of distilled water, adjusted to pH 7.0 to eliminate the phenolic acids, and then with 100 mL of ethyl acetate to elute the fraction of catechins and oligomeric procyanidins (FCO). The fraction of polymeric procyanidins (FPP) was finally eluted with 150 mL of methanol. Each fraction was evaporated to dryness under vacuum, dissolved to a concentration of 100 mg/mL with dimethyl sulfoxide, and stored at −20 °C until needed. The composition of the fraction has been shown elsewhere.29) Each fraction was used to treat the 3T3-L1 cells at final concentrations of 50 and 100 mg/mL starting at day 0 of cell differentiation.

Cell culture and differentiation of the 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured as previously described with minor modifications.30) The 3T3-L1 preadipocytes were briefly maintained to confluence at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich Co., St. Louis, MO, USA) containing 10% bovine serum (Sigma-Aldrich Co., St. Louis, MO), at two d post-confluence (designated day 0 of cell differentiation), adipocyte differentiation was induced with a mixture of methylisobutylxanthin (0.5 mM; Sigma-Aldrich Co., St. Louis, MO, USA), dexamethasone (1 μM; Sigma-Aldrich Co., St. Louis, MO, USA), and insulin (1 μg/mL; Sigma-Aldrich Co., St. Louis, MO, USA) in DMEM containing 10% fetal bovine serum (FBS, Sigma-Aldrich Co.). On day 2, this medium was replaced with DMEM containing 10% FBS and insulin only. On day 4 and thereafter, the medium consisting of DMEM and 10% FBS was subsequently replaced with a fresh medium at 2-d intervals.

Visualization and quantification of the lipids. On day 6, the cells were washed with phosphate-buffered saline (PBS) and stained with Oil Red O for 20 min, according to the method reported elsewhere.31) The TG contents were determined by using commercially available kits and were normalized by the protein concentration. Photomicrographs were taken after the cells had been air-dried. The protein content in the cell lysates was quantified by using a commercially available kit (Bio-rad Co., Hercules, CA, USA) according to the manufacturer’s protocol. The optical density was normalized by the protein content.

mRNA expression analysis. The mRNA expression levels of CCAAT/enhancer-binding protein α (CEBPα), acetyl-CoA carboxylase (ACC), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), glucose transporter-4 (GLUT4), and leptin were analyzed by real-time PCR, using a StepOne Plus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) and Taqman probe-based gene expression analysis (Applied Biosystems, Carlsbad, CA, USA). Total RNA from the cells was extracted by using the Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) under RNase-free conditions. Total RNA was reverse-transcribed by using Moloney murine leukemia virus (M-MLV) reverse transcriptase (a high-capacity reverse transcription kit, Applied Biosystems, Carlsbad, CA, USA). The resulting cDNA was added to a Master Mix containing the forward and reverse primers and Taqman probe-based probes, in a total volume of 25 μL. The reaction conditions were as follows: 2 min at 50 °C, followed by 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The mRNA expression was normalized by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the housekeeping gene expression levels were calculated as the average of the CEBPα, ACC, HSL, LPL, GLUT4, and leptin mRNA expression levels.

Statistical analyses. Data were expressed as mean ± SE values (n = 6) and were subjected to an analysis of variance (ANOVA) by SAS software (SAS Institute, Cary, NC, USA). Significant differences between treatment means were determined by using Duncan’s multiple-range test, the significance of differences being defined as the p < 0.05 level.

Results

The concentration distribution of polyphenols and procyanidins was as shown elsewhere.29) It was noticeable that the fraction of polymeric procyanidins (FPP) contained a significantly large amount of total flavonoids as well as total polymeric procyanidins. No cytotoxicity was apparent at any treatment concentration used (data not shown).

Lipid accumulation was observed and quantified as the TG content in 3T3-L1 adipocytes treated with each fraction at 50 and 100 μg/mL. All the treatments significantly reduced the lipid accumulation in differentiated adipocytes when compared to that of the control at both concentrations tested (Fig. 1). Lipid accumulation was reduced by 19% of the control level. Among the treatments, FPP was the most effective for reducing the total triglyceride accumulation in the cells (by 57.1% and 19.0%, respectively).

The mRNA level of the molecular markers related to adipocyte differentiation also supported this observation. The FPP treatment significantly decreased the level of CEBPα mRNA (Fig. 2A) at both concentrations. The CPE treatment also decreased the CEBPα mRNA level at both concentrations, while the FCO treatment was not effective. Significantly decreased level of leptin mRNA was expected since that is a factor representative of adiposity (Fig. 2B). It was notable that no significant differences in leptin mRNA expression were apparent, except with the 100 μg/mL FPP treatment.

Grape seed extract (GSE) did not influence lipolysis in the 3T3-L1 adipocytes, which was apparent from the lack of effect on the expression of HSL, in terms of mRNA level, which is the key enzyme for lipolysis32) (Fig. 3A). The mRNA level of LPL was significantly decreased by the CPE and FPP treatments at respective concentrations of 50 and 100 μg/mL. A reduced lipid uptake from lipoprotein was one of the reasons responsible for the lowered lipid accumulation by treating the 3T3-L1 cells. The expression level of LPL mRNA was significantly reduced by both CPE and FPP (Fig. 3B). Interestingly, the CPE treatment exhibited a significant effect at both a high concentration (100 μg/mL) and low concentration (50 μg/mL), while the FPP treatment only showed a significant effect at a high concentration (100 μg/mL). LPL is known to be an enzyme responsible for lipid uptake,33) and CPE and FPP seem to have had an inhibitory effect on lipid uptake with the 3T3-L1 model. Those results in conjunction with the effect of the treatment on HSL and LPL mRNA expression indicate...
that lipolytic enzyme activity was not involved in the anti-obesity effects of CPE and FPP from defatted grape seed meal.

A decreased expression of GLUT4 mRNA, which implies a change in glucose metabolism, was apparent in the CPE (100 μg/mL) and FPP (100 μg/mL)-treated groups (Fig. 4A). This may also have contributed to the decreased lipid accumulation in the treated 3T3-L1 adipocytes. Uncoupling protein 2, a key marker for energy expenditure, was increased by the CPE (100 μg/mL) and FPP (50 and 100 μg/mL) treatments (Fig. 4B). By combining the evidence from observations on the lipid and glucose metabolism, CPE as well as FPP significantly reduced lipid accumulation in the 3T3-L1 adipocytes by lowering the level of lipid synthesis, reducing the glucose and lipid uptake, and increasing the energy expenditure.

**Discussion**

Since an inhibition of fat accumulation by polyphenols has been notably suggested, there is the potential...
for procyanidins being used as a functional anti-obesity substance. Defatted grape seed meal is a rich source of such monomeric phenolic compounds as catechin, epicatechin and epicatechin gallate, and of dimeric, oligomeric, and polymeric procyanidins which are known to provide such benefit as an anti obesity effect.27) Abundant data supporting the possible control mechanism for fat metabolism are available that have proposed the relationship between AMPK and acetyl CoA carboxylase.30) The production of malonyl-CoA, which is a key substrate for building fatty acids, is governed by the activity of ACC. AMPK phosphorylates ACC, resulting in its deactivation, according to the level of AMP in the system. The contribution of procyanidins to lipid metabolism may also be linked controlling each enzyme at the transcriptional level, although that has yet to be discussed and confirmed. We have previously demonstrated a cytoprotective effect on PC 12 cells of the extract from grape seed oil meal. This cytoprotective effect was postulated to be due to radical scavenging. It can be pointed out that the functional content of the byproduct was good enough for practical use.28)

It has been suggested that leptin secretion has to be determined to evaluate the effect instead of quantifying mRNA expression in this model. Leptin secretion has been well correlated to fat accumulation in the 3T3-L1 model.30 GSE did not influence lipolysis in 3T3-L1 adipocytes as was apparent by no effect on the mechanism for fat metabolism are available that have been suggested as a plausible solution for the metabolic syndrome have been highlighted for past decades.

Since the proanthocyanidins from grape seed have been suggested as a plausible solution for the metabolic syndrome,39,40 procyanidins originating from grape seed are becoming popular as a functional food substance for treating various diseases.

We tested and confirmed in this study the effect of the biological activities of oligomeric and polymeric procyanidins from defatted grape seed meal. It is suggested from these initial results that further studies are desirable to focus on the anti-obesity effect of a purified extract of defatted grape seed meal.

References