Fucosterol inhibits adipogenesis through the activation of AMPK and Wnt/ β -catenin signaling pathways

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Abstract Fucosterol is a sterol constituent primarily derived from brown algae. Recently, the anti-adipogenic effect of fucosterol has been reported; however, its molecular mechanism remains to be studied. Fucosterol effectively upregulated the phosphorylations of both adenosine monophosphate (AMP)-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC), and downregulated the expression levels of lipogenesis-related factors. Moreover, fucosterol activated the major components of the Wnt/ β -catenin signaling pathway, including β -catenin, disheveled 2 (DVL2), and cyclin D1 (CCND1), whereas it inactivated glycogen synthase kinase 3 β (p-GSK3 β) by stimulating its phosphorylation. In the presence or absence of fucosterol, the adipogenic transcriptional factors [peroxisome proliferator activated-receptor γ (PPAR γ), CCAAT/enhancer binding protein α (C/EBP α), and sterol regulatory element binding protein-1c (SREBP-1c)] were upregulated by the inhibition of AMPK by compound C or the knockdown of β -catenin by siRNA. Overall, these data demonstrate that fucosterol prevents adipogenesis by mediating both AMPK- and Wnt/ β -catenin-signaling pathways.

Keywords: fucosterol, adipogenesis, AMPK, Wnt/ β -catenin, 3T3-L1 preadipocytes

Introduction

Obesity is a chronic metabolic syndrome resulting from a variety of reasons including endocrine function, metabolic rate, genetic, nutritional, medicinal, and other environmental factors (1). The hallmark of obesity is an adipose tissue abnormalities with increased adipocyte number and larger adipocyte size through adipocyte differentiation and increased triacylglycerol deposition in the cytoplasm (2).

Adipogenesis is a differentiation process from preadipocytes to mature adipocytes (3). The most important adipogenic transcriptional regulators, such as peroxisome proliferator activated-receptor γ (PPAR γ), CCAAT/enhancer binding protein α (C/EBP α), and sterol regulatory element binding protein-1c (SREBP-1c), are mainly expressed during the differentiation process. These adipogenic transcriptional factors are closely correlated with the expression of numerous lipid metabolizing enzymes such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (4,5). Thus, these transcriptional factors have been suggested as therapeutic targets to modulate lipid metabolism as well as obesity.

AMP-activated protein kinase (AMPK) is a crucial mediator of the cellular energy responses in that it preserves a constant supply of ATP for metabolic processes and is considered to be the sensor for

maintaining the energy balance in most mammalian cells as well as throughout the whole body (6,7). It is also well reported that AMPK potentially promotes adipocyte fatty acid oxidation and regulates lipid metabolism. AMPK activation depends on the phosphorylation of its subunit at Thr172 by AMPK kinases (AMPKKs), and the resulting activated AMPK downregulates adipogenesis by preventing the expression of adipocyte master transcription factors and inhibits the fatty acid synthesis through suppression of ACC and FAS (6,8,9). Therefore, AMPK is considered as a metabolic target for ameliorating obesity and its related metabolic diseases.

The Wnt/ β -catenin signaling pathway controls many biological processes, such as cell proliferation and differentiation during embryonic development and homeostasis in adults (10). It is associated with several diseases, including cancer, neuronal disease, and osteoporosis, and has been reported to restrain adipogenesis (10,11). This canonical signaling pathway prevents adipogenic differentiation through the ubiquitination of β -catenin by destructing the glycogen synthase kinase (GSK)3 β -Axin-adenomatous polyposis coli (APC) complex (11–13). Wnt molecule activates the signaling by binding to the membrane co-receptors such as lipoprotein receptor related protein (LRP) 5/6 and frizzled (FZD), leading to the interaction between disheveled (DVL) and Axin. The interaction causes the degradation of the GSK3 β -Axin-APC complex, and thereby β -catenin

leads to the upregulation of its target gene cyclin D1 (CCND1), which inhibits the adipogenic transcriptional factors. The downregulation of PPAR γ and C/EBP α suppresses the adipogenic differentiation from preadipocytes to mature adipocytes (13,14).

Phytosterols found in grains, vegetables, fruits, and berries have been shown to possess serum cholesterol-lowering, anti-inflammatory, antioxidant, and anticancer activities (15-17). Several reported studies have demonstrated that phytosterols prevent the risk of obesity-related metabolic diseases (18,19). Fucosterol (Fig. 1), a phytosterol primarily founded in brown algae, has been reported to exhibit physiological activities such as anti-fungal, anti-oxidant, anti-photoaging, anti-cancer, anti-diabetic, anti-inflammatory, anti-hyperglycemic, and anti-hyperlipidemic activities (20-27). Recently, its anti-adipogenic effect was also reported (28); however, its molecular mechanism remains to be studied. This study demonstrates that the suppressive effect of fucosterol on adipocyte differentiation is controlled by the activation of both AMPK- and Wnt/ β -catenin-signaling pathways.

Materials and Methods

Chemical reagents Fucosterol was supplied by Santa Cruz (CA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were supplied by Hyclone Laboratories, Inc. (Logan, UT, USA). Bovine calf serum (BCS) was supplied by Gibco (Grand Island, NY, USA). Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), and MTT, protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibody against β -catenin was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Primary antibodies, namely, phosphorylated AMPK (p-AMPK), AMPK, phosphorylated ACC (p-ACC), ACC, SREBP-1c, FAS, PPAR γ , C/EBP α , phosphorylated GSK3 β (p-GSK3 β), and α -tubulin were supplied by Cell Signaling Technology (Beverly, MA, USA).

Cell culture and adipocyte differentiation Mouse 3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM containing 10% BCS and 1% antibiotics (120 units/mL penicillin and 75 μ g/mL streptomycin) under humidified atmosphere (5% CO₂ and 37°C). For the induction of adipocyte differentiation, induction media (MDI: 0.5 mM IBMX and 0.2 μ M DEX, and 1.7 μ M insulin in DMEM containing 10% FBS) with fucosterol was treated to two-day post-confluent preadipocytes (designated day 0). After two days of differentiation induction (day 2), the media was transferred to a fresh medium containing DMEM with 10% FBS and 1.7 μ M insulin with fucosterol, which was refreshed every two days until the eighth day.

Cell cytotoxicity Cytotoxic effect of fucosterol on cell viability in 3T3-L1 adipocyte was assessed by the MTT colorimetric assay. 3T3-L1 preadipocytes were seeded at a density of 1×10^5 cells/well in 24-well plates. The cells were differentiated with different doses of fucosterol.

After the end of differentiation (day 8), the cell cytotoxicity was monitored by the MTT colorimetric assay according to a previously reported method (29). Cell cytotoxicity showed no significant toxicity when the cells were treated with fucosterol concentration up to 10 μ M (data not shown). The following experiments were conducted with fucosterol concentrations below 10 μ M.

Western blot assay 3T3-L1 adipocytes were lysed using RIPA buffer (Elpis Biotech, Daejeon, Korea) supplemented with a protease inhibitor cocktail. Total protein concentrations were quantified using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The protein was analyzed using a Western blot assay according to a previously reported method (29). The primary antibodies against phosphorylated AMPK (p-AMPK), AMPK, phosphorylated ACC (p-ACC), ACC, SREBP-1c, FAS, PPAR γ , C/EBP α , β -catenin, phosphorylated GSK3 β (p-GSK3 β), and α -tubulin were used in a 1:1,000 dilution. The bound primary antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA) (1:5,000 dilution). Antibody signals were visualized using the enhanced chemiluminescence (ECL) detection solution (Amersham Biosciences, Little Chalfont, UK) and scanned and analyzed using the G:BOX EF imaging system (Syngene, Cambridge, UK) and the Gene Snap program.

Reverse transcription-polymerase chain reaction (RT-PCR) Total RNAs extracted from the 3T3-L1 adipocyte were analyzed by conducting the reverse transcription-polymerase chain reaction (RT-PCR) according to a previously reported method (29). The following primer pairs (Bioneer, Daejeon, Korea) were used (Table 1). PCR amplifications were conducted in the Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The products of PCR were separated through 1.5% agarose gel electrophoresis and detected using the G:BOX EF imaging system (Syngene) and the Gene Snap program.

AMPK inhibition by compound C Two days after achieving confluency, 3T3-L1 preadipocytes were treated with 5 μ M of AMPK inhibitor compound C (Calbiochem, San Diego, CA, USA) or serum-free medium for 30 min. The cells were differentiated using previously described adipocyte differentiation protocol for eight days. Total protein extract was prepared for the Western blot analysis.

β -Catenin knockdown by siRNA transfection Two days after achieving confluence, 3T3-L1 preadipocytes were starved in a serum-free medium for 1 h. The cells were transfected with 60 nM of β -catenin siRNA or control siRNA (Santa Cruz) using the Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) transfection reagent. After 9 h of transfection, the cells were induced to differentiate using previously described adipocyte differentiation protocol for eight

Table 1. Primer sequences used in RT-PCR analysis

Origin	Gene	Direction	Sequence (5'–3')
Mouse	β -Catenin	Forward	GCC AAG TGG GTG GTA TAG AG
		Reverse	CTG GGT ATC CTG ATG TGC
	DVL2	Forward	GCT TCC ACA TGG CCA TGG GC
		Reverse	TGG CAC TGC TGG TGA GAG TCA CAG
	CCND1	Forward	AAA ATC GTG GCC ACC TGG AT
		Reverse	CAT CCG CCT CTG GCA TTT TG
	PPAR γ	Forward	CCT GTT GAC CCA GAG CAT GG
		Reverse	CGA GTG GTC TTC CAT CAC GC
	C/EBP α	Forward	CTG CCC CTC AGT CCC TGT C
		Reverse	GTT CCT TCA GCA ACA GCG G
	β -Actin	Forward	CCA CAC CTT CTA CAA TGA GC
		Reverse	TGA GGT AGT CAG TCA GGT C

days. Total RNA extract was prepared for RT-PCR.

Statistical analysis Data presents the mean $\pm$ standard deviation (SD). Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Sheffe's test or unpaired t-test conducted with the statistical program SPSS 21 (SPSS Inc., Chicago, IL, USA). $^{###}p<0.01$, $^{*}p<0.05$, and $^{**}p<0.01$ were considered to be statistically significant.

Results and Discussion

Fucosterol enhances the protein expression of the AMPK signaling pathway To determine whether fucosterol improves the AMPK signaling pathway by its phosphorylation, a Western blot analysis was conducted to examine the protein expression level. The expression of p-AMPK and its immediate substrate p-ACC were increased (Fig. 2A), whereas the expression of SREBP-1c and FAS, the factors related with lipogenesis, were decreased in fucosterol-treated cells (Fig. 2B). These results showed that fucosterol effectively activated AMPK and inactivated ACC by upregulating the phosphorylation of ACC, leading to the inhibition of fatty acid synthesis genes.

AMPK acts as a key metabolic master switch in many tissues, and it can be the target of obesity-related disorders (6,7). ACC, the direct substrate of AMPK, is a crucial enzyme in fatty acid metabolism and is one of the fat-specific genes stimulated by PPAR γ (4,5). One of the key roles of AMPK is to control fatty acid oxidation as well as lipogenesis by modulating the expression of lipogenesis-related gene and mediating adipocyte differentiation and maturation (6-9). SREBP-1c is the transcription factor involved in lipid metabolism and regulates the enzymes involved in triglyceride synthesis such as ACC and FAS (6,8,9). These results suggest that fucosterol activates AMPK and inhibits lipid accumulation and fatty acid synthesis through the inactivation of SREBP-1c, ACC, and FAS.

Fig. 1. Chemical structure of fucosterol.

Compound C-mediated inhibition of AMPK mediates the anti-adipogenic effect of fucosterol

The inhibitory effect of fucosterol on the differentiation of adipocytes was assessed by staining with Oil Red O. The colorimetric quantification of Oil Red O exhibited that intercellular lipid accumulation resulting from adipogenesis was significantly inhibited by fucosterol treatment in 3T3-L1 adipocyte (data not shown). The result was consistent with that of a previous report (28). To evaluate the mechanism of AMPK action in relation to the anti-adipogenic effect of fucosterol, compound C, a selective inhibitor of AMPK, was treated to downregulate the AMPK signaling pathway. Compound C suppressed the phosphorylation of AMPK and enhanced that of the key adipogenic transcription factors. However, fucosterol increased the expression of AMPK and decreased that of PPAR γ , C/EBP α , and SREBP-1c in the presence of compound C (Fig. 3). The results confirm that the modulation of the AMPK signaling pathway by fucosterol treatment inhibits adipocyte differentiation by preventing the expression of adipogenic transcription factors.

Adipogenesis, the mechanism which by preadipocytes differentiate into adipocytes, is triggered by the cascade of major adipogenic transcription factors (3). PPAR γ , the predominant adipogenic transcription factor, controls adipogenesis and promotes multiple lipogenic genes such as ACC and FAS (4). C/EBP α is essential to promote the adipogenic program and activation of PPAR γ (5). Compound-C-mediated AMPK inhibition showed that the inhibition resulted in the recovered expression of adipogenic transcription factors. The results demonstrate that fucosterol requires the activation of AMPK signaling for preventing adipogenesis and lipid metabolism. It has been reported that some phytochemicals such as 4-hydroxyderricin, xanthoangelol, dieckol, and ursolic acid also inhibit adipogenesis by modulating the AMPK signaling pathway (30-32). These reports support that the AMPK signaling pathway acts as the target for anti-adipogenesis. Our results also show that fucosterol-upregulated AMPK activation contributes to the prevention of adipogenesis through suppression of lipid accumulation.

Fucosterol upregulates the Wnt/ β -catenin signaling pathway components

To investigate whether the activation of Wnt/ β -catenin signaling is involved in the anti-adipogenic effect of fucosterol, western blot and RT-PCR were employed to evaluate the molecular

Fig. 2. Effect of fucosterol on the AMPK signaling pathway. (A) The protein levels of p-AMPK, AMPK, p-ACC, and ACC were measured by Western blot. (B) The protein levels of SREBP-1c and FAS were measured by Western blot. α -Tubulin was used as the internal loading control.

Fig. 3. Effect of fucosterol on adipogenic differentiation factors in the presence or absence of AMPK inhibitor. (A) The cells were treated with 5 μ M fucosterol in the presence or absence of compound C. The protein levels of p-AMPK, PPAR γ , C/EBP α , and SREBP-1c were analyzed by Western blot. α -Tubulin was used as the internal loading control. (B) Data are presented as the mean $\pm$ SD (% of control) of triplicate independent experiments. [#] p < 0.05 and ^{##} p < 0.01 were considered statistically significant by unpaired t-test.

mechanism. Fucosterol effectively enhanced the protein expression of β -catenin and p-GSK3 β (inactivated form) (Fig. 4A). The gene expression levels of β -catenin, DVL2, and CCND1 were effectively upregulated by fucosterol treatment in 3T3-L1 adipocyte (Fig. 4B and 4C). This demonstrates that fucosterol upregulated the Wnt/ β -catenin signaling pathway-related factors.

The canonical signaling pathway is known to be involved in the coordination of cell proliferation, differentiation, and development, affecting diseases such as cancer and osteoporosis. Recently, the signaling pathway has also been established as a key regulator of adipogenesis (11,12,15). GSK3 β , existing as the GSK3 β -Axin-APC complex, is identified as a negative regulator of Wnt/ β -catenin signaling pathway. DVL2 is the only activated member of DVL family and can disrupt the complex (11,33). Fucosterol increased the expression levels of p-GSK3 β and DVL2, suggesting that fucosterol inactivated GSK3 β by the upregulation of DVL2. Upregulated β -catenin by DVL2 can be translocated into nucleus and β -catenin leads to the activation of its target gene CCND1. Fucosterol stimulates the gene expression levels of β -catenin and its target genes such as CCND1 and DVL2, confirming that fucosterol can promote the Wnt/ β -catenin signaling by modulating the related components.

Fucosterol regulates adipogenic differentiation through activation the Wnt/ β -catenin signaling pathway To determine the correlation between the Wnt/ β -catenin signaling pathway and the inhibition effect of fucosterol on adipogenesis, β -catenin siRNA was treated into the cells. The gene expression of β -catenin and CCND1 was downregulated, whereas essential adipogenic transcription factors were upregulated in β -catenin knockdown cells in comparison with control siRNA cells (Fig. 5). Furthermore, the gene expression levels of β -catenin, CCND1, PPAR γ , and C/EBP α was reversed by fucosterol treatment in 3T3-L1 adipocyte. The data indicated that β -catenin knockdown leads to a reversed expression of adipogenic transcription factors. Accordingly, fucosterol inhibits adipogenesis through downregulation of the adipogenic transcription factors by mediating the canonical Wnt/ β -catenin signaling pathway.

Some phytochemicals such as epigallocatechin gallate and desmethylicaritin were also reported to suppress the differentiation of adipocytes through stimulation of the canonical Wnt/ β -catenin signaling pathway (33,34). These previous studies support that Wnt/ β -catenin signaling pathway is the treatment target of adipogenesis. These results also confirm that fucosterol confers an inhibitory effect on adipogenic differentiation by upregulating the elements of Wnt/

Fig. 4. Effect of fucosterol on the Wnt/β-catenin signaling pathway. (A) The protein levels of β-catenin and p-GSK3α were measured by Western blot. α-Tubulin was used as the internal loading control. (B) The mRNA expression of β-catenin, DVL2, and CCND1 was measured by RT-PCR. β-Actin was used as an internal loading control. (C) Data are presented as the mean±SD (% of control) of triplicate independent experiments. * $p<0.05$ and ** $p<0.01$ were considered statistically significant by ANOVA followed by Sheffe's test.

Fig. 5. Effect of fucosterol on adipogenic differentiation factors in the presence or absence of β-catenin siRNA. (A) The cells were treated with or without β-catenin siRNA. The mRNA levels of β-catenin, CCND1, PPARγ, and C/EBPα were detected by RT-PCR. β-Actin was used as the internal loading control. (B) Data are presented as the mean±SD (% of control) of triplicate independent experiments. # $p<0.05$ and ## $p<0.01$ were considered statistically significant by unpaired t-test.

β-catenin signaling pathway.

Collectively, fucosterol inhibited adipocyte differentiation by downregulating the adipogenic transcriptional factors and lipid metabolism related factors by activating the AMPK signaling pathway in combination with the upregulation of Wnt/β-catenin signaling pathway. It is anticipated that fucosterol will acts as a therapeutic natural agent for the treatment or prevention of obesity and obesity-induced metabolic disease, although further studies are necessary to evaluate its anti-obesity activity *in vivo*.

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