Hexacosanol reduces plasma and hepatic cholesterol by activation of AMP-activated protein kinase and suppression of sterol regulatory element-binding protein-2 in HepG2 and C57BL/6J mice

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ABSTRACT

Policosanols have hypocholesterolemic activity; however, the molecular mechanism of the policosanol effects is currently poorly characterized. We hypothesized that hexacosanol, a policosanol compound derived from barley sprout, may decrease cellular and plasma cholesterol levels; we thus investigated the hypocholesterolemic activity and mechanism of hexacosanol on both hepatocytes and high-fat induced obese C57BL/6J mice. The reduction of total cholesterol, free cholesterol, and cholesteryl ester concentrations was confirmed in hexacosanol-stimulated hepatocytes (−38%, −33%, and −53%, respectively). Plasma, hepatic cholesterol concentrations, and hepatic steatosis were significantly reduced in high-fat fed mice orally administered with hexacosanol (0.7 mg/kg body weight a day) for 8 weeks compared with those of vehicle-fed control mice (−15% and −40%, respectively). Hexacosanol in fact bound to the allosteric regulation site of AMP-activated protein kinase (AMPK)-β subunit and thus activated AMPK that inhibited the activity of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase by inhibitory phosphorylation. In addition, activation of AMPK by hexacosanol induced hepatic autophagy activity, which could further reduce hepatic lipid accumulation. Alternatively, hexacosanol suppressed the nuclear translocation and activation of sterol regulatory element-binding protein-2 (SREBP-2), a key transcription factor in cholesterol biosynthesis. These results collectively suggest that hexacosanol is a major hypocholesterolemic compound in barley sprouts with regulation of AMPK activation and SREBP-2 suppression. These suppress 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase at both mRNA expression and protein activity levels. In conclusion,

Keywords: Hexacosanol, Hypocholesterolemia, AMPK, SREBP-2, HMG-CoA reductase

Abbreviations: AMPK, AMP-activated protein kinase; ATG, autophagy-related gene; CRP, C-reactive protein; Dil, 1,10-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; ER, endoplasmic reticulum; FPLC, fast protein liquid chromatography; HDL, high-density lipoprotein; HFD, high-fat diet; HMGCR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; IL-6, interleukin-6; INSIG, insulin-induced protein-1; LC3, anti-microtubule-associated protein 1A/1B-light chain 3; LDL, low-density lipoprotein; SCAP, SREBP cleavage-activating protein; SREBP, sterol regulatory element-binding protein; TNFα, tumor necrosis factor-α.

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1. **Introduction**

Hypercholesterolemia is a major cause of cardiovascular disease. The reduction of low-density-lipoprotein (LDL) cholesterol levels is a primary preventive and therapeutic strategy for the control of cardiovascular disease [1,2]. Statin, an inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), a rate-limiting enzyme in cholesterol biosynthesis, is an effective hypocholesterolemic drug [1,2]. However, some individuals do not respond to statin therapy; others show significant adverse effects including rhabdomyolysis, proteinuria, and nephropathy [3]. Therefore, the use of natural substances has been suggested as an alternative strategy for the reduction of LDL cholesterol levels.

Natural compounds often regulate multiple targets to show net physiological effects. An understanding of molecular basis of antihypercholesterolemic effects is necessary to assess the effects of natural substances. In the cellular cholesterol metabolism, HMGCR is a prerequisite target, which can be regulated at both transcriptional and posttranslational levels. Transcription of HMGCR is regulated primarily by sterol-regulatory element-binding protein-2 (SREBP-2) [4,5]; thus, the suppression of SREBP-2 activity should reduce the gene expression of the HMGCR. In addition, the HMGCR enzyme activity is suppressed by AMP-activated protein kinase (AMPK) by inhibitory phosphorylation at Ser872 [6]. Alternatively, AMPK also phosphorylates SREBP-2 that inhibits its nuclear translocation and activation [7]. Collectively, activation of AMPK and/or suppression of SREBP-2 potentially lead to the reduction of LDL cholesterol levels.

Policosanols, aliphatic primary alcohols, widely exist in plant cuticle including spouts of crops, sugar cane, rice bran [8], and edible vegetable oils [9-11]. Policosanols are known as AMPK activators that regulate cellular homeostasis in lipid metabolism with unknown mechanism [12]. Several clinical trials have suggested the efficacy of policosanols from sugarcane. In meta-analysis, the consumption of 10 to 20 mg/d policosanol lowers total cholesterol levels, LDL cholesterol levels, and systolic blood pressure, while increasing the ratio of high-density lipoprotein (HDL) cholesterol to LDL cholesterol [13]. However, other studies attempting to confirm the cholesterol-lowering efficacy of policosanols from sugarcane have failed in both animal [14,15] and human models [16-18]. Therefore, the hypocholesterolemic effects of sugarcane policosanols remain elusive.

We have reported that barley sprout extract containing policosanols revealed potent hypocholesterolemic effects both in vitro and in vivo [19]. Barley sprout extract contains hexacosanol (C26 saturated primary fatty alcohol, Fig. 1A) as a major policosanol compound [20]. In contrast, policosanols from sugarcane include octacosanol as a main component. We hypothesized that hexacosanol is an active hypocholesterolemic phytochemical. Previous studies using rat models demonstrated that hexacosanol exerts an antidiabetic effect; however, its effect on cholesterol metabolism is unknown [21,22]. To test this hypothesis, we performed in vitro and in vivo studies to confirm the hypocholesterolemic effects of hexacosanol and investigate the underlying molecular mechanism(s).

2. **Methods and materials**

2.1. **Materials**

Hexacosanol (purity >97%), simvastatin (purity >97%), and hexane (purity >95%) were purchased from Sigma-Aldrich (St Louis, MO, USA). Isopropanol (purity >99%) and ethanol (purity >99%) were obtained from Merck (Darmstadt, Germany). A-769662 (purity >98%) from Cayman Chemical (Ann Arbor, MI, USA) was used in this study.

2.2. **In vitro AMPK kinase activity assay**

Effect of hexacosanol on AMPK activity was quantified with luminescent ATP/ADP detection assay (Promega, Madison, WI, USA) [23]. Briefly, either hexacosanol or positive control (A-769662) was added to the reaction buffer containing AMPK proteins (α1/β1/γ1) and ATP (150 μmol/L) and was incubated for 1 hour at room temperature. The kinase reaction was terminated by stop solution. The produced ADP was quantified after addition of kinase detection reagent with luminescent ATP/ADP detection assay (Promega, Madison, WI, USA) was used in this study.

2.3. **Cell culture and treatment**

HepG2 cells (Korean Cell Line Bank, Seoul, Korea) were grown in Dulbecco modified Eagle medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C, 5% CO2. Cells were seeded at a concentration of 5 × 10^5 cell/well into 6-well culture plates and stimulated with metformin (1 mmol/L), simvastatin (2 μmol/L), or hexacosanol (12.5, 25, and 50 μmol/L) for 24 or 4 hours for phosphor-protein extraction. All experiments with cultured cells were repeated in triplicate [19].

2.4. **Cellular lipid staining and quantification**

Lipid-loaded hepatocytes were stimulated with hexacosanol for 24 hours, and cellular lipid concentrations were quantified after lipid extraction. Fluorescence 1,10-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen, Carlsbad, CA, USA) was used for staining of intracellular lipids in the cells as described elsewhere [24]. Briefly, HepG2 cells were incubated with DiI for 20 minutes at 37°C. Cells were washed with fresh media, fixed with 4% formaldehyde, and then analyzed with a confocal laser-scanning microscope.
For cellular lipid measurement, cell lysates were extracted with hexane-isopropanol mixture (2:1, vol/vol) for 30 minutes at room temperature. The solvent was evaporated using vacuum centrifugation, and the lipids were redissolved in 200 mL of 95% ethanol prior to lipid quantification. Intracellular cholesterol concentrations were quantified with cholesterol/cholesteryl ester kit (Abcam, Cambridge, MA, USA). The concentrations were normalized with cellular protein concentrations assessed with Bradford assay (Bio-rad, Hercules, CA, USA).

2.5. Animal care and experiments

Animal care and handling were performed according to protocols approved by the Animal Experimentation and Ethics Committee of Korea University (Protocol No. KUIACUC-2016-97). C57BL/6J male mice (8 weeks old, n = 6-10) were purchased from Samtako (Seoul, Korea) and maintained in specific pathogen-free facility under 12-hour light/dark cycle, constant temperature (25°C), and 50% relative humidity. The mice were fed AIN-76A–based high-fat diet (HFD) (45% and 4% of energy from fat and cholesterol, Table 1) for 4 weeks to cause the diet-induced obesity; then animals were randomly divided into 3 groups: vehicle control (phosphate-buffered saline), simvastatin (70 μg/day/kg of body weight), and hexacosanol (700 μg/day/kg of body weight) groups. This dose was calculated from the amount of 20 mg of barley sprout extract per day per 60-kg human [25]. Simvastatin and hexacosanol were dissolved in distilled water and dimethyl sulfoxide, respectively, and were orally administered by gavage for 8 weeks. During feeding, body weight and food consumption were measured twice per week. Food and water were given ad libitum. At 8 weeks of feeding, mice were

![Chemical structure of hexacosanol](https://example.com/hexacosanol_structure.png)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Weight (g/kg of diet)</th>
</tr>
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<tbody>
<tr>
<td>Casein</td>
<td>233.1</td>
</tr>
<tr>
<td>l-Cystine</td>
<td>3.5</td>
</tr>
<tr>
<td>Corn starch</td>
<td>84.8</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>116.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>201.4</td>
</tr>
<tr>
<td>Cellulose</td>
<td>58.3</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>29.1</td>
</tr>
<tr>
<td>Lard</td>
<td>206.9</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>11.7</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>15.1</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>6.4</td>
</tr>
<tr>
<td>Potassium citrate</td>
<td>19.2</td>
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<tr>
<td>Vitamin mix</td>
<td>11.7</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.3</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
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Forty-five percent of total energy was provided from fats.
anesthetized with isoflurane, and blood samples were collected by cardiac puncture and then centrifuged at 3000g for 5 minutes to collect plasma samples. Livers were snap frozen in liquid nitrogen or were fixed in 4% formaldehyde for histological analysis.

2.6. Plasma cholesterol and lipoprotein analysis

Plasma cholesterol analysis was performed every 4 weeks (0, 4, and 8 weeks). Plasma samples were collected retroorbitally after 12-hour fasting, and total, HDL, and LDL cholesterol concentrations were determined enzymatically using an automatic analyzer (Cobas C111; Roche, Basel, Switzerland). Lipoprotein profiling was analyzed using fast protein liquid chromatography (FPLC) system as described previously [26] (AKTA Purifier 10; GE Healthcare, Piscataway, NJ, USA). Pooled mouse plasma (300 μL) from 5 mice per group was injected onto the 2 tandem Superose 6 10/300 GL columns (GE Healthcare) and separated with elusion buffer containing 154 mmol/L NaCl, 1 mmol/L EDTA, and 0.02% NaN3 (pH 8.2) at a flow rate of 0.35 mL/min.

2.7. Hepatic lipid and cytokine quantification

The hepatic cholesterol and triglyceride concentrations were determined enzymatically using Cobas C111 with enzymatic quantification methods [27]. Plasma concentrations of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP) were quantified with commercial enzyme-linked immunosorbent assay kit from Novus Biologicals (Littleton, CO, USA), Biovendor R&D (Brno, Czech Republic), and Thermo Scientific (Rockford, IL, USA), respectively.

2.8. Histological analysis of liver and aorta

Liver and aorta were fixed in 4% formaldehyde and stained with hematoxylin and eosin (H&E). The images were acquired from microscope (Carl-Zeiss), and we analyzed by Axio Imager M1 software (Carl-Zeiss) in the Department of Pathology, Anam Hospital of Korea University (Seoul, Korea) [28].

2.9. Total RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted from hepatocytes and C57BL/6J mice liver tissues by using RNAiso Plus Kit (Takara Bio Inc, Shiga, Japan) according to the manufacturer’s instruction. Real-time polymerase chain reaction was performed with Bio-Rad iQ SYBR Green Supermix reagent and a Bio-Rad iQ5 cycler system (Richmond, CA, USA). The isolated RNA was treated with DNase prior to cDNA synthesis. Primer sequences of the investigated genes are presented in Table 2. Gene expression levels were calculated with threshold cycle [29] method according to the manufacturer’s guidelines and normalized to cyclophilin-β expression level [30].

2.10. Protein extraction and immunoblot assays

Protein samples were lysed using RIPA buffer (10 mmol/L Tris-HCl, pH 7.5; 1% NP-40; 0.1% sodium deoxycholate; 0.1% SDS; 150 mmol/L NaCl; and 1 mmol/L EDTA) supplemented with 1× protease and phosphatase inhibitor cocktail (Thermo, Fremont, CA, USA). The separated proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell Bioscience, Dassel, Germany). Nonspecific binding was blocked with 5% nonfat dry milk in TBS-T buffer for 1 hour at room temperature. The membranes were incubated with primary antibody overnight at 4°C. The monoclonal anti-AMPK, phospho-AMPK, HMGR, phospho-HMGR, precursor SREBP-2, mature SREBP-2, insulin-induced protein-1 (INSIG-1), α-tubulin, and β-actin and the secondary antibodies (anti-mouse and anti-rabbit immunoglobulin G) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against proteins involved in the autophagy pathway, including anti-microtubule-associated protein 1A/1B-light chain 3 (LC3) and autophagy-related gene 1 (ATG16L) were obtained from Novus (Littleton, CO, USA).

Upon incubation, the membranes were washed with TBS-T for 40 minutes and then incubated with appropriate secondary antibody for 1 hour at room temperature. Immunoreactivity protein bands were detected with enhanced chemiluminescence system (Animal Genetics, Seoul, Korea), visualized using a ChemiDoc XRS+ System (Bio-Rad), and quantified with Gel-Pro Analyzer software (Silk Scientific, Inc., Orem, UT, USA) [31].

2.11. Fluorescent microscopic analysis of cellular autophagy

Hepatocytes cultured in 6-well plates at 1.5 × 10^5 cells/mL were transfected with tandem fluorescent mRFP-GFP-LC3 plasmids using FuGENE HD reagent (Promega) according to the manufacturer’s instructions. Cells were fixed with 4% paraformaldehyde and stained with 4’-6-diamidino-2-phenylindole and mounted with Antifade reagent (Molecular Probes, Eugene, OR USA). Image was taken on a Zeiss LSM 5 Exciter confocal microscope (100×/1.30 oil DIC; Carl Zeiss, Jena, Germany) according to the manufacturer’s guidelines and analyzed by Zeiss LSM510 v.3.2 software (Carl Zeiss). The number of yellow puncta represents the formation of autophagosomes, whereas the number of red puncta represents autolysosomes. The numbers of yellow (autophagosomes) and red puncta (autolysosomes) were counted in 3 random areas for each group of liver sections [32].

| Table 2 – Polymerase chain reaction primer sequences |
| Gene | Sequence (5′→3′) |
| Human SREBP-2 | Forward: agg cag gct tgt aag acg aag tac tgt aca tct gaa cag ggc |
| | Reverse: tta gtt cca tta gcc tca tca tca gaa |
| Human HMGR | Forward: tcc agc gag tct ggg cta aat tag a |
| | Reverse: atg tgt aca tcg gaa cag gcg |
| Human Cyclophilin | Forward: tgg gag agy ycc tct atg |
| | Reverse: gag atg ggg acc tgt aa |
| Mouse SREBP-2 | Forward: gtc caa ttt ggc agc tca gcc |
| | Reverse: tcc agc gag tat ggg cta aat tag a |
| Mouse HMGR | Forward: gat caa atg tcc acc aca acc c |
| | Reverse: ggc cga tga cga gcc c |
| Mouse INSIG-1 | Forward: ggt gtt tgg acc tgt aac ttt gtc tg |
2.12. Statistical analyses

Data are expressed as the means ± SEM. Comparisons between groups were performed by 1-way analysis of variance followed by Tukey test. Differences with P < .05 were considered to be statistically significant.

3. Results

3.1. Hexacosanol reduces intracellular cholesterol concentrations in hepatocytes

Hypocholesterolemic effect of hexacosanol was examined in lipid-loaded HepG2 cells. Dil fluorescence of cytosolic lipid accumulation was reduced in cells stimulated with metformin (1 mmol/L), simvastatin (2 μmol/L), and hexacosanol (50 μmol/L) (Fig. 1B). Intracellular cholesterol measurements revealed that hexacosanol significantly reduced total cholesterol, free cholesterol, and cholesteryl ester concentrations by 38%, 33%, and 53%, respectively, compared with vehicle (dimethyl sulfoxide)-treated controls (P < .05). The ratio of free cholesterol to cholesteryl ester was also significantly increased in hexacosanol-stimulated cells, 3.9 vs 6.1 for control and hexacosanol, respectively, P < .29 (Fig. 1C). These results suggest that hexacosanol reduces intracellular cholesterol levels in hepatocytes.

3.2. Hexacosanol ameliorates atherosclerosis and hepatic steatosis in high-fat–fed C57BL/6J mice

The effects of hexacosanol on cholesterol and lipid metabolism were investigated in HFD-fed C57BL/6J mice orally administrated hexacosanol (0.7 mg/kg body weight per day) for 8 weeks. Food intake and body weight were not different among groups (Fig. 2). Total and LDL cholesterol concentrations were significantly reduced in hexacosanol-fed mice by 15% and 40%, respectively, compared with those in controls (Fig. 3A). Plasma lipoprotein profiling with FPLC revealed similar results showing reductions in cholesterol levels in LDL particles (Fig. 3B). Results from H&E staining of liver tissues suggested a substantial reduction of lipid accumulation in livers and arteries (Fig. 3C). Livers from hexacosanol-fed group showed a significant reduction in hepatic cholesterol by 40% (Fig. 3D). In addition, plasma levels of TNFα and CRP proteins were significantly decreased in hexacosanol-fed mice compared with those of controls approximately 40% and 32%, respectively (Fig. 3E). These results suggest that hexacosanol exerts hypocholesterolemic effects in HFD-fed mice by ameliorating symptoms of hepatic steatosis and atherosclerosis.

3.3. Hexacosanol allosterically regulates AMPK activity and induces inhibitory phosphorylation of HMGCR

Policosanols are known to activate AMPK that suppresses HMGCR activity. However, the molecular basis of the activation is not clearly understood. In vitro AMPK kinase activity assay was undertaken to examine the direct interaction and activation of AMPK with hexacosanol. Octacosanol, a major compound in sugarcane policosanols, was used as a comparison. Our data showed that both hexacosanol and octacosanol directly bound to and activated AMPK with EC50 values of 11 and 17 μmol/L, respectively (Fig. 4A). Potential hexacosanol binding site on AMPK protein was also explored by competitive assay in which hexacosanol was coincubated with AMPK agonist A-769662 or AMP. A-769662 and AMP bind to AMPK β and γ subunit, respectively, thus activating AMPK activity. Our results revealed that the EC50 value of AMP to AMPK was slight reduced to that of AMP and hexacosanol coincubation, 8 vs 32 μmol/L, respectively (Fig. 4B). These suggest that hexacosanol and AMP may have distinct binding sites on AMPK. In contrast, AMPK activity was markedly inhibited approximately 88-fold when hexacosanol was coincubated with A-769662. The EC50 value of A-769662 was 3 μmol/L compared with 264 μmol/L when coincubated with hexacosanol (Fig. 4B). These suggest that hexacosanol and A-769662 may share binding site in AMPK β subunit that blocks the effect of A-769662 by competition. Further immunoblotting analysis showed that AMPK phosphorylation at Thr172 was induced by 286% at a concentration of 50 μmol/L hexacosanol-stimulated hepatocytes (Fig. 4C) and 70% in livers from hexacosanol-fed compared with those in controls (Fig. 4D). Expectedly, the HMGCR inhibitory phosphorylation (Ser872)
was increased significantly by 205% and 135% at 50-μmol/L hexacosanol-stimulated hepatocytes and livers, respectively (Fig. 4 C and D). These results collectively suggest that hexacosanol directly binds to and activates AMPK that may reduce intracellular cholesterol accumulation through inhibitory phosphorylation at HMGCR both in vitro and in vivo.

3.4. Hexacosanol delays nuclear translocation of SREBP-2

In line with the induction of inhibitory phosphorylation on HMGCR, we quantified that the mRNA expression levels of HMGCR were reduced with borderline significance in hexacosanol group (P < .52) (Fig. 5A). These suggest that the hypcholesterolemic effect of hexacosanol could include the regulation of SREBP-2, a key transcription factor in cellular cholesterol homeostasis [5]. Although we did not observe the induction in either mRNA or precursor protein expression levels of SREBP-2 by hexacosanol (Fig. 5 B and C), the substantial reduction in the levels of mature SREBP-2 (mSREBP-2) protein was determined to be approximately 62% (Fig. 5C). The ratio of mature to precursor form of SREBP-2 tended to be decreased with borderline significance (Fig. 5C). In addition, we also measured the expression of INSIG-1, which tended to be induced approximately 78% by
hexacosanol (Fig. 5C). Induction of INSIG-1 has known to delay the endoplasmic reticulum (ER)-to-Golgi transport of SREBP-2 complex by SREBP cleavage-activating protein (SCAP) [5]. Together, our results suggest that hexacosanol may have a marginal effect on the nuclear translocation of SREBP-2.

3.5. Hexacosanol activates hepatic autophagy

Intracellular lipids are stored in lipid droplets, and reduction of intracellular cholesterol may facilitate lipid droplet degradation. Autophagy is a self-degradation process for cellular organelles including lipid droplets that reuses the intracellular constituents by lysosomal function, and autophagic pathway is stimulated by AMPK [33]. Induction of autophagy in hepatocytes suppresses lipid accumulation that protects liver steatosis and reductions in intracellular lipids [34]. We thereby investigated the effects of hexacosanol on hepatic autophagy pathway to evaluate AMPK activation by hexacosanol. Immunoblotting analysis of livers revealed that hexacosanol induced key proteins in autophagy pathway including ATG16L1 (75%) and LC3-II (77%) in hexacosanol-fed group compared with those of controls (Fig. 6A). Further fluorescence microscope image analysis of hepatocytes-transfected mRFP-GFP-LC3 tandem expression vectors assessed the formation of autophagosome and autolysosome, respectively (Fig. 6B). The formation of autophagosome (yellow puncta) and autolysosome (red puncta) was increased in the hexacosanol group compared with that in control by approximately 1.8-fold (Fig. 6B). These results suggest that hexacosanol reduces intracellular cholesterol levels in liver through induction of autophagy pathway, and this may at least in part contribute to the reduction of intracellular lipid accumulation (Fig. 7).
4. Discussion

Policosanols are natural aliphatic primary alcohols with carbon chain lengths between 24 and 34 [35]. The hypocholesterolemic effects of policosanols have been identified in various animal studies that showed the inhibition of cholesterol biosynthesis or the induction of LDL catabolism [13]. Based on these effects, policosanols, primarily isolated from sugarcane, have been used as functional foods and food supplements with hypocholesterolemic effects. However, many clinical studies have not observed significant reductions in total or LDL cholesterol levels using policosanols from sugarcane under different clinical settings [14,16-18]. Therefore, whether these compounds truly have hypocholesterolemic effects has remained somewhat controversial. A study in Cuba that examined the effects of policosanols isolated from sugar cane containing octacosanol (>60%) as a major compound reported positive results [8], whereas other studies have not observed significant hypocholesterolemic effects of policosanols from sugarcane. However, our previous study has shown that policosanols from barley sprout wax possesses strong

Fig. 5 – Hexacosanol suppresses HMGCR gene expression via suppression of nuclear translocation of SREBP-2. mRNA expression levels of HMGCR (A) and SREBP-2 (B). C, Immunoblotting analysis of precursor and mature forms of SREBP-2 and INSIG-1 protein expression levels induced by hexacosanol. The data are expressed as means ± SEM. *P values compared with controls are denoted as *P < .05 and **P < .01. n = 5. Ctr, vehicle control; Met, metformin (1 mmol/L); Sta, simvastatin (2 μmol/L); Hex, hexacosanol (12.5, 25, and 50 μmol/L).
hypocholesterolemic activities with hexacosanol as a major policosanol compound [19]. It is possible that an active compound in policosanols is hexacosanol and the low level of hexacosanol resulted in the mixed results from the clinical trials with sugarcane policosanol. In this study, we hypothesized that hexacosanol is an active hypocholesterolemic compound in policosanols. Results from this study revealed that hexacosanol is an effective hypocholesterolemic natural compound. The effects was comparable to the 10% dose of simvastatin, a potent hypocholesterolemic drug.

![Fig. 6 – Hexacosanol induces hepatic autophagy. A, Immunoblotting analysis of protein expression levels belonging to autophagy pathway in hexacosanol-fed mice livers. B, Immunofluorochemistry analysis of hepatocytes transfected with mRFP-GFP-LC3 tandem vector. Yellow puncta indicate autophagosomes and red puncta autolysosomes. The data are expressed as means ± SEM. P values compared with controls are denoted as *P < .05 and **P < .01. n = 5. Ctr, vehicle control; Sta, simvastatin (2 μmol/L); Hex, hexacosanol (50 μmol/L).](image)

![Fig. 7 – Proposed mechanism of hypocholesterolemic effects of hexacosanol in high-fat-fed mice. Hexacosanol reduces plasma and hepatic cholesterol by activation of AMPK and suppression of SREBP-2. AMPK activation by hexacosanol inhibits HMGCR through inhibitory phosphorylation. Hexacosanol delays nuclear translocation of SREBP-2 that reduces HMGCR gene expression. In addition, hexacosanol induces hepatic autophagy via AMPK activation. These collectively show hypocholesterolemic effects of hexacosanol in HFD-induced obese mice.](image)
For the first time, we demonstrated that hexacosanol derived from barley sprout directly binds to AMPK-β subunit, thereby functioning as an allosteric activator. Previous studies have shown that policosanols and octacosanol induce AMPK and HMGCR phosphorylation, but the molecular target remains unclear [12]. Our study revealed that the direct interaction and allosteric activation of AMPK with hexacosanol lead to inhibitory phosphorylation on Ser872 of HMGCR, a rate-limiting enzyme in cholesterol biosynthesis.

Together with the activation of AMPK-HMGCR signaling axis, we examined whether hexacosanol delays the nuclear translocation of SREBP-2 that suppresses HMGCR transcription. Posttranslational regulation of SREBPs has been well characterized [4,36]. SCAP is a molecular chaperone that escorts the transport of SREBP from the ER to the Golgi apparatus. INSIG-1 forms a triple complex with SCAP-SREBP in the ER membrane. During posttranslational processing of SREBP, the SCAP-SREBP complex moves from the ER to the Golgi in which S1P and S2P proteases release N-terminal peptide of SREBP, which is translocated to the nucleus and functions as an active transcription factor that regulates target gene transcription, including HMGCR [4,36]. We provided evidence that hexacosanol delays the nuclear translocation of SREBP-2, resulting in downregulation of HMGCR gene expression.

Based on our results, we propose that hexacosanol-induced INSIG-1, a key factor in the SREBP-mediated regulation, may result in the decrease of SREBP nuclear translocation. AMPK has known to positively regulate INSIG-1 expression such that the transfection of AMPKδ subunits induces INSIG-1 expression, whereas the transfection of dominant-negative AMPK suppresses the transcription of INSIG-1 [37]. However, the detailed mechanism underlying the effects of AMPK on INSIG-1 remains unclear. Further studies are required to identify the effects of AMPK on INSIG-1 mRNA and protein expression. Herein, our data showed that hexacosanol induced INSIG-1 expression and decreased levels of the active nuclear form of SREBP-2 (mSREBP-2) with borderline significance, suggesting that it may have a marginal effect on the activation of SREBP-2 by AMPK-dependent manner.

In hepatocytes, intracellular lipids are stored as lipid droplets and are catabolized by lipases to provide energy to maintain the cells. In addition, an alternative pathway has been proposed, the autophagy pathway, which is mediated by the lysosomal degradative pathway [38,39]. In this pathway, lipid droplets containing cholesteryl ester are taken up by autophagosomes that fuse to lysosomes to form an autolysosome for further degradation through acidic hydrolysis. This pathway is also controlled by AMPK through the inhibition of the rapamycin signaling axis [33]. Consistently, we explored that hexacosanol-activated AMPK upregulates major mediators involving autophagy pathway, ATG16 and LC3-II, which mainly regulate the formation of the autophagosome resulting in the reduction of hepatic lipid accumulation [40].

It has been evidenced that natural botanical compounds target multiple molecules regulating many biological pathways. Our results indicate that hexacosanol regulates AMPK and SREBP-2 resulting in hypcholesterolemic effects both in vitro and in vivo. Additionally, we also observed that the concentration of 2 key regulators of the inflammatory response, TNFα and CRP, was markedly reduced in hexacosanol-administrated mice compared with that in the control group. TNFα and CRP are involved in systemic inflammation that responds to IL6 production [41,42]. As the limitation of this study, we cannot rule out the role of IL-6 here because we could not significantly determine the reduction of IL-6 protein concentration in plasma. It is worthy to note that AMPK activation has been reported to inhibit TNFα-induced NF-κB transactivation [43,44]. These raise a possibility that hexacosanol may function as an anti-inflammatory compound in HFD-fed mice. Policosanols from pomace olive oil also suppressed the release of proinflammatory mediators, such as TNF-α, by reducing production of nitric oxide, a regulator of the inflammatory and immune reactions of macrophages. These results indicate that policosanols, including hexacosanol, may have a protective effect against inflammation [45]. The potential role of hexacosanol in anti-inflammatory effects awaits further investigation.

In summary, hexacosanol reduces plasma and hepatic cholesterol through the AMPK activation and SREBP-2 suppression that induces HMGCR inhibitory phosphorylation and suppresses the HMGCR gene expression, respectively. In addition, hexacosanol also induces hepatic autophagy, which was mediated by AMPK activation, resulting in hypcholesterolemic effects in obese mice. Consequently, our finding supports that hexacosanol intake provides metabolic benefits in HFD-induced obese mice.

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