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Anti-obesity effect of carboxymethyl chitin by AMPK and aquaporin-7 pathways in 3T3-L1 adipocytes

Chang-Suk Kong[a], Jung-Ae Kim[b], Soon-Sun Bak[c], Hee-Guk Byun[d], Se-Kwon Kim[b,c,*]

[a]Department of Food and Nutrition, College of MEdical and Life Science, Silla University, Busan 617-736, Republic of Korea
[b]Department of Chemistry, Pukyong National University, Busan 608-737, Republic of Korea
[c]Marine Bioprocess Research Center, Pukyong National University, Busan 608-737, Republic of Korea
[d]Faculty of Science and Biotechnology, Kangnung National University, Gangneung 210-702, Republic of Korea

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Abstract

The aim of this study was to investigate the anti-obesity effect of carboxymethyl-chitin (CM-chitin), a water-soluble derivative of chitin, by measuring lipid accumulation and adipogenesis related factors in 3T3-L1 adipocytes. CM-chitin was synthesized by means of carboxymethylation reaction. Its inhibitory effect on lipid accumulation was investigated by measuring triglyceride content and glycerol release level. The gene and protein levels associated with adipogenesis were determined using reverse transcriptase-polymerase chain reaction and Western blot analysis. Treatment with CM-chitin reduced triglyceride content and enhanced glycerol secretion in a dose-dependent manner. CM-chitin induced the down-regulation of adipogenesis related transcriptional factors and adipocyte specific gene promoters. Moreover, the specific mechanism by CM-chitin was confirmed by transcriptional activations of the phosphorylated adenosine monophosphate-activated protein kinase (AMPK) and aquaporin-7. These results suggest that CM-chitin exerts anti-adipogenic effect on lipid accumulation through modulations of AMPK and aquaporin-7 signal pathways.

Keywords: Adipocyte differentiation; Adipogenesis; AMPK; Aquaporin-7; Lipid accumulation

1. Introduction

Obesity predisposes a person to a variety of pathological disorders and has become a serious public health problem [1–3]. Adipocytes play a central role in regulating adipose mass and obesity in accordance with lipid homeostasis, energy balance and secreting various transcription factors [4]. 3T3-L1 preadipocytes can differentiate into mature adipocytes, which include well-developed lipid droplets. 3T3-L1 cells have been frequently used as an in vitro model to assess adipocyte differentiation, lipid accumulation studies and its cellular regulatory mechanisms [5]. The relationships between obesity and or lipid accumulation by adipocyte differentiation have been reported in several studies [5,6]. For these reasons, a number of research efforts have been conducted on 3T3-L1 adipocytes as a screening method to search for health foods/agents beneficial in controlling obesity. Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimeric enzyme composed of a catalytic α subunit and two regulatory subunits, β and γ [7]. AMPK acts as a fuel sensor in regulating glucose and lipid homeostasis in adipocytes and its activation leads to numerous metabolic changes; therefore, it is critical for controlling metabolic disorder such as obesity and diabetes [8,9]. Aquaporin-7 modulates adipocyte glycerol permeability and controls lipid accumulation or its size as a glycerol channel in adipocytes [10]. The adipose aquaporin-7 has been associated with development of obesity [10].

Chitin is a naturally abundant mucopolysaccharide found in the shell of crustaceans, the cuticle of insects and the cell wall of some fungi and microorganisms [11]. Chitin is extensively acetylated, consists of *N*-acetyl-D-glucosamine units and is insoluble in water as well as in acid. This low solubility of chitin is considered to be a major limiting factor for its commercial applications in biomedical, food and chemical industries. As a result, a number of chemical trials on the modification and application of chitin have been conducted to develop its water-soluble derivatives [11,12]. Carboxymethylation and deacetylation reactions were used in converting them to water-soluble forms, which may be useful in biomedical, foods, agricultural and other related industries [13–15].

In this study, the inhibitory effect of carboxymethyl-chitin (CM-chitin) on adipocyte differentiation was investigated by measuring lipid accumulation level and expression level of adipocyte marker gene in 3T3-L1 cells. Moreover, we examined whether AMPK and aquaporin-7 signals were critical for the anti-obesity function of CM-chitin.

2. Materials and methods

2.1. Synthesis of CM-chitin

Synthesis of CM-chitin was carried out using the method developed by Tokura et al. [16]. Briefly, chitin (1 g) was suspended in 15 M NaOH (12.5 ml) and stirred for 60 min

* Corresponding author. Tel.: +82 51 629 7094; fax: +82 51 629 7099.
E-mail address: sknkim@pknu.ac.kr (S.-K. Kim).

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at 20°C. The alkali-chitin was recovered by filtration on a sintered glass filter funnel under suction. The materials were resuspended in a solution of monochloroacetic acid (1.5 g) in isopropanol (12.5 ml) and followed by stirring for 5 h at 20°C. Afterward, 50 ml of distilled water was added and then pH was adjusted to 7.0 with 11 M HCl solution. After 24 h, the reaction mixtures were filtered and then the filtrates were precipitated with 5 volumes of acetone. The CM-chitin was reprecipitated, desalted by electrodialysis and lyophilized (Fig. 1A). The degree of substitution was determined using an infrared spectrometer (Shimadzu IR-408, Tokyo, Japan) (Fig. 1B).

2.2. Cell culture and adipocyte differentiation

Mouse 3T3-L1 preadipocytes were grown to confluence in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. At 1 day postconfluence (designated "Day 0"), cell differentiation was induced with a mixture of methylisobutylxanthine (final concentration [finc.] 0.5 mM), dexamethasone (finc. 0.25 μM) and insulin (finc. 5 μg/ml) in DMEM containing 10% FBS. After 48 h (day 2), the induction medium was removed and replaced by DMEM containing 10% FBS supplemented with insulin (finc. 5 μg/ml) alone. This medium was changed every 2 days. The adipocyte differentiation was induced up to Day 6. The CM-chitin was then used to treat culture medium of adipocytes at day 6. After treatment with the CM-chitin for 24 h, the adipocytes were lysed for triglyceride, reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis. Cytotoxicity of the CM-chitin was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Any significant toxic effect was not observed on the cells treated with the CM-chitin up to a concentration of 200 μg/ml (data not shown).

2.3. Morphological changes based on Oil-red O staining

Cells were washed gently twice with phosphate-buffered saline (PBS), fixed with 3.7% fresh formaldehyde in PBS for 1 h at room temperature, and stained with filtered Oil-red O solution (60% isopropanol and 40% water) for at least 1 h. After staining, the Oil-red O staining solution was removed and the plates were rinsed with water and dried. Images of lipid droplets in 3T3-L1 adipocytes were collected by an Olympus microscope (Tokyo, Japan).

2.4. Measurement of triglyceride content

Cellular triglyceride contents were measured using a commercial triglyceride assay kit (Triglyzyme-V, Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions. Adipocytes differentiated for 6 days were treated with the CM-chitin at concentrations of 50, 100 and 200 μg/ml in 12-well plates for 24 h (from Days 6–7). The cells were washed twice with, scraped in 75 μl of homogenizing solution (154 mM KCl, 1 mM EDTA, 50 mM Tris, pH 7.4) and sonicated to homogenize the cell suspension. The residual cell lysate was centrifuged at 3000 rpm for 5 min at 4°C to remove the fat layer. The supernatants were assayed for triglyceride content and protein content.

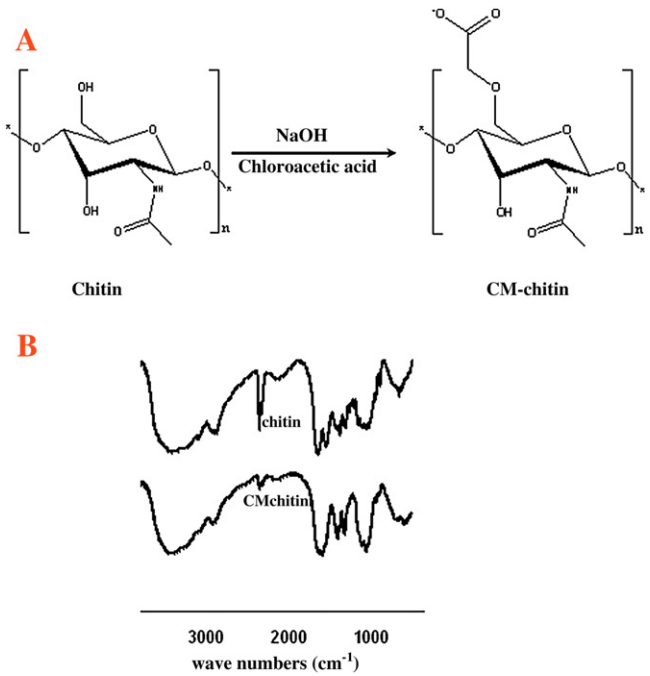

Fig. 1. Synthesis pathway (A) and FT-IR spectra (B) of CM-chitin by carboxymethylation reaction.

Triglyceride was normalized to protein concentration determined by bovine serum albumin as a standard. Results were expressed as milligrams of triglyceride per milligram of cellular protein.

2.5. Measurement of glycerol level

The glycerol level was determined using the enzymatic reagent, free glycerol reagent (Sigma-Aldrich, St. Louis, MO, USA), directed by the protocol of GPO-TRINDER (Sigma-Aldrich).

2.6. RNA extraction and reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from 3T3-L1 adipocytes using Trizol reagent (Invitrogen, Carlsbad, CA, USA). For synthesis of cDNA, 2 μg of RNA was added to RNase-free water and oligo (dT), denaturated at 70°C for 5 min and cooled immediately. RNA was reverse transcribed in a master mix containing 1× RT buffer, 1 mM dNTPs, 500 ng oligo

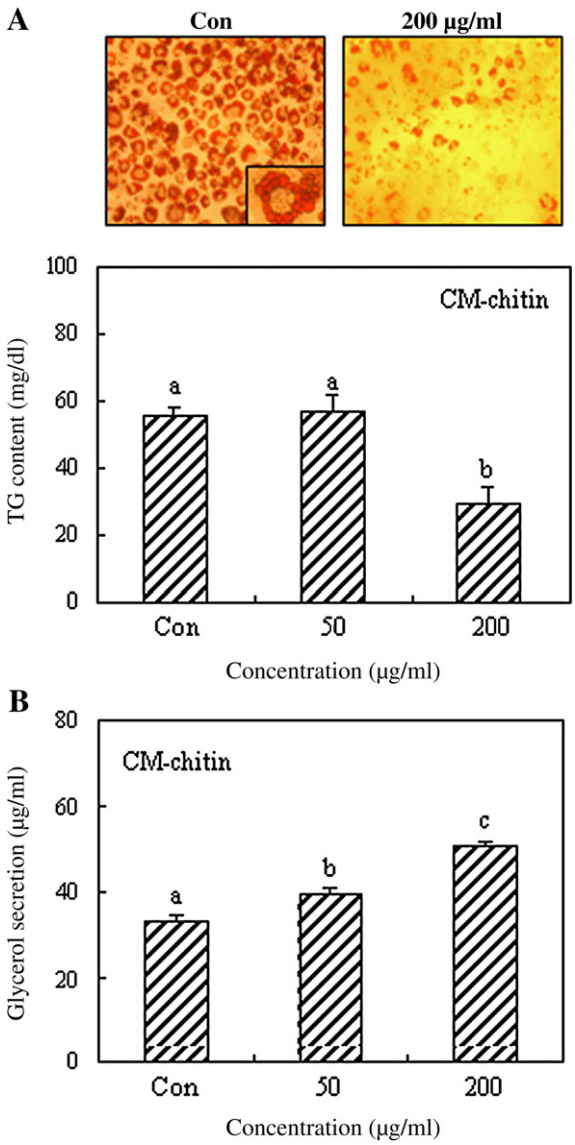

Fig. 2. Effect of CM-chitin on lipid accumulation and lipolytic activity in 3T3-L1 adipocytes. Confluent 3T3-L1 preadipocytes were differentiated into adipocytes for 6 days (from Day 0 to Day 6) and treated with different concentration of CM-chitin for 24 h. (A) The lipid accumulation was measured by triglyceride assay and representative image based on Oil-red O staining was presented. (B) The lipolytic activity of CM-chitin was determined by measuring glycerol levels secreted in medium. $^{a-c}$Means with the different letters are significantly different ($P<.05$) according to Duncan's multiple range test. Con, fully differentiated control adipocytes (0.5 mM methylisobutylxanthine, 0.25 μM dexamethasone and 5 μg/ml insulin).

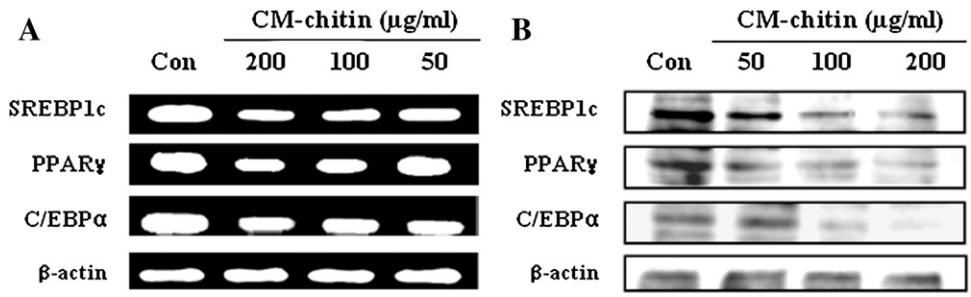

Fig. 3. Effect of CM-chitin on PPARγ, SREBP1c and C/EBPα gene (A) and protein (B) expressions in 3T3-L1 adipocytes. Confluent 3T3-L1 preadipocytes were differentiated into adipocytes for 6 days (from Day 0 to Day 6) and treated with different concentration of CM-chitin for 24 h.

(dT), 140 U M-MLV reserve transcriptase and 40 U RNase inhibitor at 42°C for 60 min using an automatic thermocycler (Biometra GmbH, Goettingen, Germany). The target cDNA was amplified using the following sense and antisense primers: forward 5′-TTT-TCA-AGG-GTG-CCA-GTT-TC-3′ and reverse 5′-AAT-CCT-TGG-CCC-TCT-GAG-AT-3′ for PPARγ; forward 5′-TGT-TGG-CAT-CCT-GCT-ATC-TG-3′ and reverse 5′-AGG-GAA-AGC-TTT-GGG-GTC-TA-3′ for SREBP1c; forward 5′-TTA-CAA-CAG-GCC-AGG-TTT-CC-3′ and reverse 5′-GGC-TGG-CGA-CAT-ACA-GTA-CA-3′ for C/EBPα; forward 5′-TCA-CCT-GGA-AGA-CAG-CTC-CT-3′ and reverse 5′-AAT-CCC-CAT-TTA-CGC-TGA-TG-3′ for adipocyte fatty acid binding protein (FABP4); forward 5′-TGC-CTC-TGC-CTT-GAT-CTT-TT-3′ and reverse 5′-GGA-ACC-GTG-GAT-GAA-CCT-AA-3′ for fatty acid transport protein (FATP1); forward 5′-TTG-CTG-GCA-CTA-CAG-AAT-GC-3′ and reverse 5′-AAC-AGC-CTC-AGA-GCG-ACA-AT-3′ for FAS; forward 5′-TCC-AAG-GAA-GCC-TTT-GAG-AA-3′ and reverse 5′-CCA-TCC-TCA-GTC-CCA-GAA-AA-3′ for LPL; forward 5′-GGA-TCA-GGT-TTT-GTG-GTG-CT-3′ and reverse 5′-TTG-TGG-CCC-ATA-AAG-TCC-TC-3′ for Leptin; forward 5′-AAG-GAT-CCT-GCA-CCT-CAC-AC-3′ and reverse 5′-CCT-CTG-CTG-AAG-GGT-TAT-CG-3′ for Perilipin; forward 5′-GAG-GGA-CAC-ACA-CAC-ACC-TG-3′ and reverse 5′-CCC-TTT-CGC-AGC-AAC-TTT-AG-3′ for hormone-sensitive lipase (HSL); forward 5′-AGC-CAT-GTA-CGT-AGC-CAT-CC-3′ and reverse 5′-TCC-CTC-TCA-GCT-GTG-GTG-GTG-AA-3′ for β-actin. The amplification cycles were carried out at 95°C for 45 s, 60°C for 1 min and 72°C for 45 s. After 30 cycles, the PCR products were separated by electrophoresis on 1.5% agarose gel for 30 min at 100 V. Gels were then stained with 1 mg/ml ethidium bromide visualized by UV light using AlphaEase gel image analysis software (Alpha Innotech, San Leandro, CA, USA).

2.7. Western blot analysis

Western blotting was performed according to standard procedures. Briefly, cells were lysed in RIPA buffer (Sigma-Aldrich) at 4°C for 30 min. Cell lysates (25 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech, England, UK), blocked with 5% skim milk and hybridized with primary antibodies (diluted 1:1000, Cell Signaling Technology, Inc., Danvers, MS, USA). After incubation with horseradish-peroxidase-conjugated secondary antibody (Cell Signaling Technology) at room temperature, immunoreactive proteins were detected using a chemiluminescent ECL assay kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Western blot bands were visualized using a LAS3000 Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

2.8. Statistical analysis

Data were expressed as mean±S.E. (n=3). Differences between the means of the individual groups were assessed by one-way analysis of variance with Duncan's multiple range tests. Differences were considered significant at P<.05. The statistical software package SAS v9.1 (SAS Institute, Cary, NC, USA) was used for the analysis.

3. Results

3.1. Effect of CM-chitin on lipid accumulation

To explore the effect of CM-chitin on adipocyte differentiation, differentiated adipocytes were treated with various concentration of CM-chitin at end of the differentiation period. As a major market of adipogenesis, lipid accumulation was quantified by measuring triglyceride content in differentiated adipocytes lysate and morphological changes based on Oil-red O staining (Fig. 2A). Treatment with CM-chitin reduced triglyceride content, which the contents were approximately 57, 57 and 29 μg/ml at the concentrations of 0, 50 and 200 μg/ml, respectively (P<.05).

In order to examine whether the reduction in triglyceride content was associated with lipolysis, the lipolytic activity of CM-chitin in 3T3-L1 adipocytes was determined by measuring the glycerol level secreted in the medium at the concentrations of 0, 50 and 200 μg/ml. Treatment with CM-chitin increased glycerol secretion according as the concentrations. The secreted glycerol levels were approximately 33, 39 and 51 μg/ml at the concentrations of 0, 50 and 200 μg/ml, respectively (Fig. 2B).

3.2. Effect of CM-chitin on adipogenic specific gene and protein expressions

RT-PCR and Western blot analysis were conducted to investigate whether CM-chitin affected the expression of adipogenic transcription factors (Figs. 3A and B). Treatment with CM-chitin reduced size and intensity of the lytic zone on regulation of peroxisome proliferator-activated receptor-γ (PPARγ), differentiation-dependent factor 1/sterol regulatory element-binding protein 1c (SREBP1c) and CCAAT/enhancer-binding proteins (C/EBPα) genes, compared to fully differentiated control adipocytes. The inhibitory effects of CM-chitin exhibited a dose-dependent pattern. Treatment with CM-chitin also suppressed the expression levels of PPARγ, SREBP1c and C/EBPα proteins.

We further investigated whether the CM-chitin regulates the adipogenic target genes such as FABP4, FATP1, fatty acid synthase (FAS), lipoprotein lipase (LPL) and leptin (Fig. 4). Treatment of adipocytes with CM-chitin significantly down-regulated the expressions of FABP4, FATP1, FAS, LPL and leptin genes in a dose-dependent manner, compared to fully differentiated adipocytes without sample treatment.

3.3. Effect of CM-chitin on lipolysis-related gene expression

Since treatment with CM-chitin decreased triglyceride levels and increased glycerol secretion, we investigated whether CM-chitin affects the regulation of lipolytic response genes (Fig. 5). Treatment with CM-chitin down-regulated perilipin gene in a dose-dependent manner while treatment with CM-chitin up-regulated *HSL* gene at a concentration of 200 μg/ml compared to fully differentiated adipocytes.

3.4. Effect of CM-chitin on regulations of aquaporin-7 and AMPK

Whether the inhibition of adipocyte differentiation by CM-chitin was mediated by AMPK activation, we compared the protein levels of phosphorylated AMPK (α and β) and its substrate, acetyl-CoA carboxylase (ACC) using western blot analysis (Fig. 6A and B). CM-chitin enhanced AMPK activation in a dose-dependent manner. Consistent with enhanced AMPK activity, phosphorylation of ACC was enhanced after treatment with CM-chitin. In order to confirm this activation, 3T3-L1 cells were pretreated with AMPK inhibitor

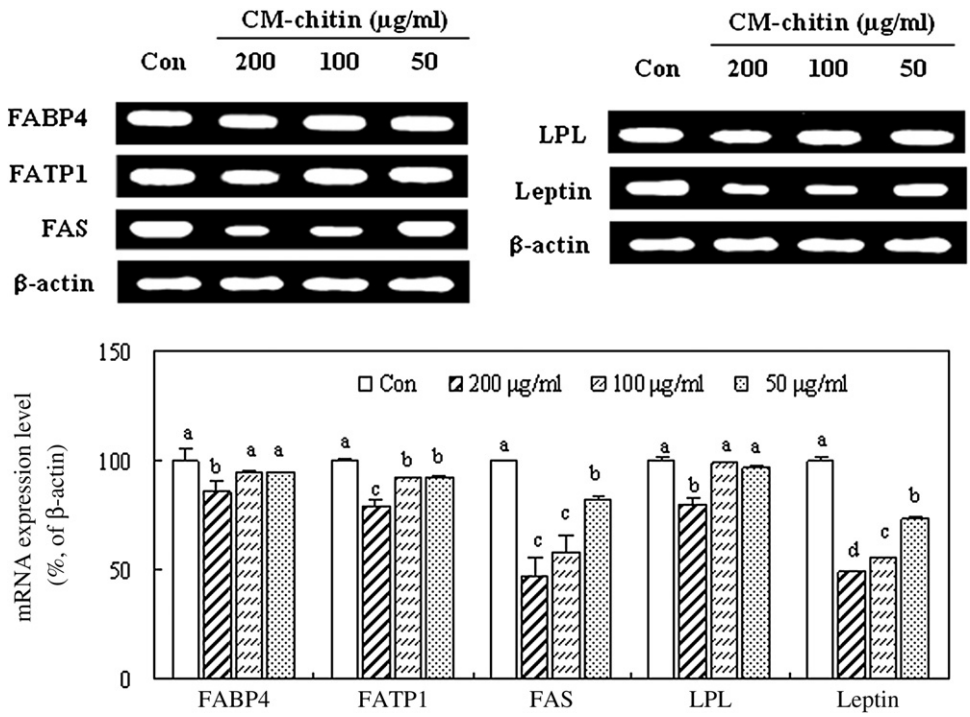

Fig. 4. Effect of CM-chitin on FABP4, FATP1, FAS, LPL and leptin gene expressions in 3T3-L1 adipocytes. Confluent 3T3-L1 preadipocytes were differentiated into adipocytes for 6 days (from Day 0 to Day 6) and treated with different concentration of CM-chitin for 24 h. [a–d]Means with the different letters at each gene are significantly different ($P<.05$) by Duncan's multiple range test. Con: fully differentiated control adipocytes (0.5 mM methylisobutylxanthine, 0.25 μM dexamethasone and 5 μg/ml insulin).

compound C and exposed to CM-chitin. The AMPK activation by CM-chitin was inhibited by pretreatment with AMPK inhibitor. In addition, the role of the aquaporin-7 in the inhibition of adipocyte differentiation by CM-chitin was assessed using western blot analysis (Fig. 6C). Treatment with CM-chitin activated aquaporin-7 expression level compared to fully differentiated adipocytes.

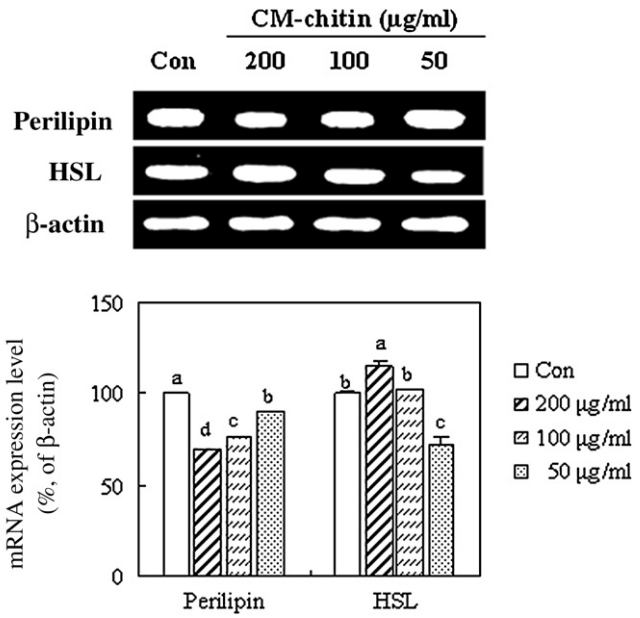

Fig. 5. Effect of CM-chitin on perilipin and *HSL* gene expressions in 3T3-L1 adipocytes. Confluent 3T3-L1 preadipocytes were differentiated into adipocytes for 6 days (from Day 0 to Day 6) and treated with different concentration of CM-chitin for 24 h. [a–d]Means with the different letters at each gene are significantly different ($P<.05$) by Duncan's multiple range test.

4. Discussion

Obesity is defined as a heavy accumulation of fat in the body's fat cells to such a serious degree, which can induce the development of

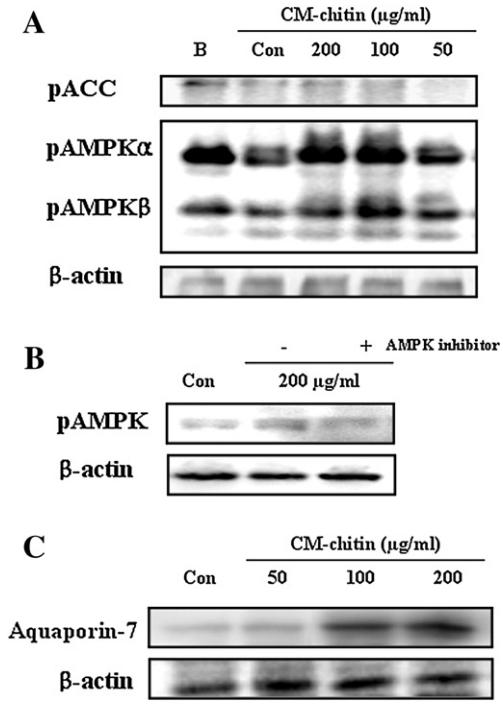

Fig. 6. Effect of CM-chitin on AMPK and aquaporin-7 activations. 3T3-L1 adipocytes were treated with CM-chitin at the indicated concentration and AMPK (A and B) and aquaporin-7 (C) activations were determined by western blot analysis. (B) Cells were pretreated with 20 μM of AMPK inhibitor compound C for 30 min and exposed to CM-chitin. B, confluent preadipocytes.

metabolic disorders such as Type 2 diabetes, hypertension, cancer, cardiac injury, osteoarthritis and heart disease [2,8]. While a number of chemical trials on the modification and application of chitin have been conducted to develop its water-soluble derivatives [12], antiobesity effects of CM-chitin are poorly understood. In this study, we investigated antiobesity effect of CM-chitin, as a water-soluble derivative of chitin, in 3T3-L1 adipocytes. Lipid accumulation and expression of several genes associated with adipogenesis and lipolysis during adipocyte differentiation were examined at the end of differentiation period in cultured 3T3-L1 adipocytes. In our study, treatment of cultured 3T3-L1 adipocytes with CM-chitin significantly blocked lipid accumulation in a dose-dependent manner ($P<.05$) (Fig. 2). Morphological assay showed that culture of 3T3-L1 under differentiation conditions remarkably induced many lipid droplets, indicating lipid accumulation, compared to the adipocyte treated with CM-chitin. Glycerol production is increased during lipolysis in adipocytes [17]. Treatment with CM-chitin enhanced glycerol secretion in adipocytes. This meant that CM-chitin can effectively inhibit adipogenesis through decrease of intracellular lipid formation and induced lipolysis through hydrolysis of triglyceride.

Adipocyte differentiation is a highly controlled process, which induces a series of programmed changes in adipose-specific gene expression. The specific transcription factors such as PPARγ, SREBP1c and C/EBPα are regulated in this process. They are well known to be critical activators for adipogenesis and can accelerate differentiation of preadipocytes into adipocytes [10]. While 3T3-L1 adipocytes undergoing differentiation remarkably induced the expressions of PPARγ, SREBP1c and C/EBPα, a significant down-regulation of these transcriptional factors was induced by treatment with CM-chitin in gene and protein levels (Fig. 3). PPARγ, SREBP1c and C/EBPα are known to be regulated in early change stage during differentiation of preadipocytes into adipocyte, which can activate the adipocyte specific gene promoters such as FABP4, FATP1, FAS, LPL and leptin [18]. FABP4 and FATP1 play important roles in the obesity pathway linked to fatty acid metabolism [19]. Decreased expressions of FABP4 and FATP1 are in accordance with a decrease in utilization of fatty acid and a decreased transport of fatty acid into the cell, respectively [19]. FAS has been known as one of the lipogenic enzymes which facilitate the synthesis and cytoplasmic storage of massive amounts of triglyceride [20]. LPL acts as catalyst in hydrolysis reactions of triglyceride, in which plasma triglyceride is metabolized to free fatty acids for triglyceride synthesis with adipose cells [21]. Leptin is exclusively secreted in proportion to their triglyceride stores in adipocytes [22]. In our results, treatment with CM-chitin induced down-regulation of FABP4, FATP1, FAS, LPL and leptin (Fig. 4). Further, in order to examine whether the blocking of lipid accumulation by CM-chitin is associated with lipolysis, perilipin and HSL expression levels were compared (Fig. 5). Presence of CM-chitin down-regulated perilipin and up-regulated HSL level at a concentration of 200 μg/ml, but not too high, compared to fully differentiated adipocytes. Perilipin acts as a protective coating from lipid droplets in adipocytes and the body's natural lipases such as HSL [23]. Perilipin expression is elevated in obese animals and humans. HSL mediate the hydrolysis of triglyceride into free fatty acids and glycerol in lipolysis metabolism [23].

To elucidate the molecular mechanism underlying anti-adipogenesis and lipolysis, we investigated the effect of CM-chitin on activations of phosphorylated AMPK, its substrate and aquaporin-7. AMPK activation can regulate the adipogenic target genes involved in diverse pathways in such tissues as adipose tissue, liver, muscles and hypothalamus [24]. AMPK modulation has developed as a major target for the controlling obesity and diabetes [25–27]. Aquaporin-7 acts as a glycerol channels for glycerol release from adipocytes and facilitates the secretion of glycerol from adipocytes, a process that contributes to the regulation of lipid accumulation factor [28]. The regulation of aquaporin-7 expression in adipose tissue has served as one of the determining factors for control of lipid accumulation and glucose homeostasis [10]. Treatment with CM-chitin activated AMPKα and AMPKβ and the activation of these kinases led to the phosphorylation of their substrate, ACC. Also, treatment with CM-chitin regulated aquaporin-7 expression in protein levels.

In differentiated adipocytes, triglyceride synthesis and hydrolysis are stimulated according to the energetic status [10,29]. Triglycerides are synthesized by esterfication reaction between carboxylic acid in free fatty acids and trifunctional alcohol in glycerol in adipocytes. The anti-obesity effect of CM-chitin may be related to in the hydrophilic properties of carboxyl group and the hydrophobic properties of N-alkyl group and pyranoze moieties. The partially negative charge in the molecule could potentially contribute in chelating to a cation [30]; thereby, it could act as chelating ligands. That is, acetyl and carboxylmethyl groups in CM-chitin are involved in electron-withdrawing group, especially the negatively charged carboxyl groups easily bind to hydroxyl groups in glycerol generated from cells. In turn, it might influence slightly the inhibition of adipogenesis and induction of the subsequent triglyceride lipolysis in adipocytes for maintaining lipid metabolism.

In conclusion, our results revealed that CM-chitin suppressed adipogenesis and induced lipolysis in 3T3-L1 cells through the modulations of aquaporin-7 and AMPK pathways, which are critical for anti-obesity effect of CM-chitin.

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