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Sulfation of chitosan oligomers enhances their anti-adipogenic effect in 3T3-L1 adipocytes

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ABSTRACT

Inhibition of adipogenesis and lipid accumulation has a very crucial role to prevent obesity. Low molecular weight (LMW) chitosan is known to inhibit fat accumulation and adipogenesis. (N,O)-sulfated chitosan is a sulfation derivative of chitosan oligomers, and its anti-obesity effect is not yet reported. In this study, it has been reported that (N,O)-sulfated chitosan significantly decreased lipid accumulation, an indicator for adipogenesis, in differentiating 3T3-L1 preadipocyte cells. Furthermore, mRNA expressions and protein levels of key adipogenic markers such as peroxisome prolifetor-activated receptor (PPAR)-γ and CCAAT-enhancer binding protein (C/EBP)-α were considerably decreased by (N,O)-sulfated chitosan treatment. As a consequence, sulfation of LMWC remarkably increased its effect of adipogenesis inhibition, adding (N,O)-sulfated chitosan high potential to be utilized as a bioactive agent in food and pharmaceutical industry.

1. Introduction

Chitin is linear β-(1→4) glycan composed of 2-acetamido-2-deoxy-D-chitosan is a cationic polysaccharide prepared by N-deacetylation of chitin in the presence of alkali. The fact that chitosan is a very abundant polysaccharide, as well as nontoxic and biodegradable, encourages the development of new applications for chitosan (Muzzarelli, 1993; Jeon & Kim, 2000).

Chitosan oligomers are hydrolysis products obtained by chemical or enzymatic treatment of chitosan and contain up to 10 units. They are typically soluble in aqueous solutions. With its high absorption rate *in vivo* systems, use of chitosan oligomer is expected to be more efficient than chitosan and chitin. Moreover, it has been reported that chitosan oligomer possesses a number of biological activities such as antitumor, antifungal, antimicrobial, antiviral, fat lowering and free radical scavenging activities (Kim & Rajapakse, 2005).

Chemical modifications of low molecular weight (LMW) chitosan attracted much attention because such approach would enhance or add biological activities while keeping the natural backbone of the chitosan and the biochemical properties of the introduced group. In this respect, sulfated derivatives of chitin and chitosan have been reported for their various biological activities (Jayakumar, Nwe, Tokura, & Tamura, 2007). In order to develop blood anticoagulants, sulfation of chitosan has been the most preferred modification because of mechanism of action similarity with heparin (Muzzarelli & Giacomelli, 1987; Park, Je, Jung, Ahn, & Kim, 2004; Vikhoreva et al., 2005). Furthermore, sulfated LMW chitosan oligomers (LMWC) have been researched for their distinct anti-HIV-1 activity.

White adipose tissue is a major energy reserve in higher eukaryotes and storing triacylglycerol in intervals of energy excess, its mobilization during the energy requirement are its primary purposes. However, initiation and endurance of obesity occur pursuant to not only hypertrophy of adipose tissue but also differentiation of preadipocytes into adipocytes. This mechanism of adipogenesis is triggered by adipose tissue hypergenesis (Spiegelman & Flier, 1996). Obesity is closely correlated to the prevalence of diabetes (Schwartz & Porte, 2005), cardiovascular disease (Spiegelman & Flier, 2001), hypertension and cancer (Calle & Kaaks, 2004). Differentiated adipocytes secrete obesity-related factors called adipokines. Plasma leptin, tumor necrosis factor (TNF)-α and non-esterified fatty acid levels are all elevated in obesity and play a role in causing insulin resistance (Leong & Wilding, 1999). Therefore, suppression and regulation of obesity can be achieved by inhibiting adipocyte differentiation and forcing adipocytes to lipolysis to reduce accumulated white adipose tissue (Langin, 2006; Yamauchi et al., 2001). Thus, the increased control of the harmful effects on

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the accumulation of adipose tissue and its metabolism contribute to the search for a better understanding of the molecular mechanisms of obesity.

For *in vitro* adipocyte differentiation, function and adipose tissue accumulation studies, 3T3-L1 is a well-known and frequently used preadipocyte cell line (Green & Kehinde, 1975). 3T3-L1 cells undergo an adipogenic differentiation when treated with a cocktail, including dexamethasone, methylisobutylxanthine and insulin. After acquisition of adipocyte function and morphology, 3T3-L1 cells accumulate microscopically detectable triglyceride droplets and express adipocyte-specific proteins. Adipocyte differentiation is coordinately regulated by several transcription factors. CCAAT element binding protein (C/EBP)-β and -γ, and acetyl-CoA and sterol response element binding protein 1 (ADD1/SREBP1) are important transcription factors in lipid metabolism. These factors are active during the early differentiation process and induce the expression and/or activity of the peroxisome proliferator-activated receptor-γ (PPAR-γ) which is the essential coordinator of the adipocyte differentiation process. Differentiation into adipocytes and conservation of adipocyte-like characteristics are controlled by these transcription factors, leading to elevation of adipocyte specific gene expression, secretion of adipokines as well as accelerated adipose and glucose metabolism (Morrison & Farmer, 1999). Therefore, altered expression of these transcription factors might underlie the development of disorders characterized by increased adipose tissue.

Jae et al. (2008) suggested that chitosan oligomers exert inhibitory effect on adipocyte differentiation in 3T3-L1 cells. Further, it has been known that some sulfated polysaccharides such as fucoidan and monosaccharides such as glucosamine. LMWCamine inhibit adipocyte differentiation (Ahn et al., 2006; Kong, Kim, & Kim, 2009). However, sulfated derivatives of chitosan oligomers have not been studied for their antiobesity properties to date. Therefore, the aim of this study is to investigate the effect of sulfated derivative of LMWC on adipogenic differentiation of 3T3-L1 pre-adipocytes. Beyond this, we compared the plain chitosan oligomer and its sulfated derivative based on their effects on adipocyte differentiation whether structural modification by sulfation would improve its anti-adipogenic activity.

2. Experimental

2.1. Reagents

Chitosan oligomers of low molecular weight (below 1 kDa) were kindly donated by Kitto Life Co. (Seoul, Korea). Dulbecco's Modified Eagle Medium (DMEM), antibiotics, fetal bovine serum (FBS) were purchased from Gibco BRL, Life Technology (NY, USA). Primers and other RT-PCR chemicals were obtained from Promega (Madison, WI, USA). Chemicals used in (N,O)-sulfated chitosan synthesis were purchased from Junsei Chemical Co. (Tokyo, Japan). All other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA)

2.2. Preparation of (N,O)-sulfated chitosan

Our previous method (Park et al., 2004) was adapted to chitosan oligomers (below 1 kDa) with a slight modification. Basically, 10 g of LMWC was dispersed in 100 ml of dimethylformamide (DMF) and put on a heating magnetic stirrer. Solution was heated up to 60 °C while adding 8 ml of chlorosulfonic acid dropwise for 30 min. The resulting solution was stirred for 4 more hours at 60 °C. After 4 h of stirring, 500 ml acetone was added in order to precipitate the solution. The precipitate was dissolved in 250 ml distilled water and then dialyzed exhaustively against distilled water (DW), using an electrodialyzer (Micro Acilyzer G3, Asahi Chemical Industry Co., Tokyo, Japan) and lypholized. Dialyzed samples were freeze-dried, gained as light brown fluffy powder, dissolved in distilled water, filtered through 0.2 μM filter and used for *in vitro* experiments.

2.3. Cell culture

3T3-L1 cells was cultured and maintained in DMEM supplemented with 10% (v/v) FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in 95% air–5% CO_2. Cells were subcultured by detaching with trypsin–EDTA solution 2–3 times in every week at about 70–80% confluency. Only cells at the passage number below 15 were used for experiments.

2.4. Adipogenic differentiation

3T3-L1 pre-adipocyte cells were maintained in DMEM containing 10% FBS and cultured in 6- or 12-well plates. Following two days of preincubation of the confluent cells, differentiation was induced by initiation of a differentiation medium (DM) containing DMEM supplemented with 10% FBS, 0.25 μM dexamethasone (DEX), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 5 μg/ml insulin. After two days of DM incubation, cell culture medium was changed to feeding medium (FM) containing DMEM with 10% FBS and 5 μg/ml insulin. Cells were fed with fresh FM every 48 h. After observation of full differentiation, cells were kept at normal cell culture medium containing DMEM with 10% FBS. For further experiments, cells were harvested at day 6 of differentiation. In order to determine the effect of (N,O)-sulfated chitosan and LMWC on adipocyte differentiation, different concentrations of LMWC and (N,O)-sulfated chitosan were treated at differentiation day 1 to cell medium together with DM, and refreshed with each medium change until the cell harvest at day 6.

2.5. Cell viability assay

The cytotoxicity levels of (N,O)-sulfated chitosan on non-differentiated and differentiated 3T3-L1 cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as reported in our previous studies with a slight modification (Karadeniz, Artan, Kong, & Kim, 2010). In case of differentiated cells, same procedures were carried out in 12-well plates due to hindrance of differentiation in small wells of 96-well plate. Briefly, 3T3-L1 cells were induced to differentiate in 12-well plates. Previous procedure was applied by modifying the amounts of MTT and DMSO to 1 ml instead of 100 μl. The Relative cell viability was calculated as a percentage of untreated cells' viability that was taken as control.

2.6. Oil Red O staining

Measurement of lipid droplets accumulated in the cells was achieved by Oil Red O staining as previously described (Ramirez-Zacarias, Castro-Munozledo, & Kuri-Harcuch, 1992). Cells on day 6 of differentiation in 6-well plates were washed twice with phosphate buffer saline (PBS) prior to fixing with 3.7% (250 μl) formaldehyde (Jinsei Co., Japan). Lipid droplets in the cytoplasm were stained with Oil Red O solution. After the staining process, Oil Red O solution was removed and wells were washed with water and dried. Images of stained cells were obtained by a fluorescent microscope (CTR 6000; Leica, Wetzlar, Germany). Later on, stain was eluted with isopropanol and quantified by measuring the absorbance at 500 nm. Triglyceride accumulation of the cells, which was calculated as stain amount, was evaluated as the relative percentage of untreated control cells.

2.7. Triglyceride assay

Cells were washed with PBS, and homogenized with buffer containing 154 mM KCl, 50 mM Tris and 1 mM EDTA. Triglyceride content of lysed cells was measured using a Triglyceride Assay Kit (Shinyang Chemical, Republic of Korea) according to manufacturer's instructions. Amount of intracellular triglyceride was standardized to the total protein amount of cells measured by protein assay kit (BioRad Laboratories, Hercules, CA).

2.8. Glycerol assay

Glycerol release into the medium of cultured and differentiated 3T3-L1 cells were measured. 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).

2.9. Reverse transcription polymerase chain reaction (RT-PCR) assay

Total RNA was extracted using a Trizol reagent as reported in manufacturer's manual. Two micrograms of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) to give cDNA and a PCR reaction was performed using specific primers as reported previously (Kong et al., 2009).

2.10. Western blot analysis

Differentiated cells in the presence or absence of different concentrations of (N,O)-sulfated chitosan at day 6 were lysed in the lysis buffer (RIPA buffer, Sigma Chemical Co., St. Louis, MO, USA) and cell lysates were used for immunoblot analysis. Total protein amount of cell lysates was measured by protein assay kit (BioRad Laboratories, Hercules, CA, USA) and the exact amounts of total protein was used for standard Western blot analysis procedures reported earlier (Karadeniz et al., 2010). The immunoreactive proteins were detected by chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, England, UK) according to the manufacturer's instructions. The resulting protein bands were visualized using the LAS3000 image analyzer (Fujifilm Life Science, Tokyo, Japan).

2.11. Statistical analysis

Statistical significance of experiments was determined and expressed as mean of three independent experiments ± standard deviation. Differences between the means of the individual groups were analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System, SAS v9.2 (SAS Institute, Cary, NC, USA) with Duncan's multiple range tests. The significance of differences was defined at the $p < 0.05$ level.

3. Results and discussion

3.1. Preparation of (N,O)-sulfated chitosan

(N,O)-sulfated chitosan was synthesized as light-brown, fluffy and water-soluble powder and structural modifications were confirmed by FT-IR, which gave the specific absorption peaks of C–O–S, S=O, and S–N at 800, 1240 and 1350 cm^{-1}, respectively, in comparison to unmodified LMWC (Fig. 1). Elemental analysis data also represented the percentages of N, C, S, H and O of (N,O)-sulfated chitosan as 5.16%, 26.06%, 11.01%, 4.57% and 53.07%, respectively.

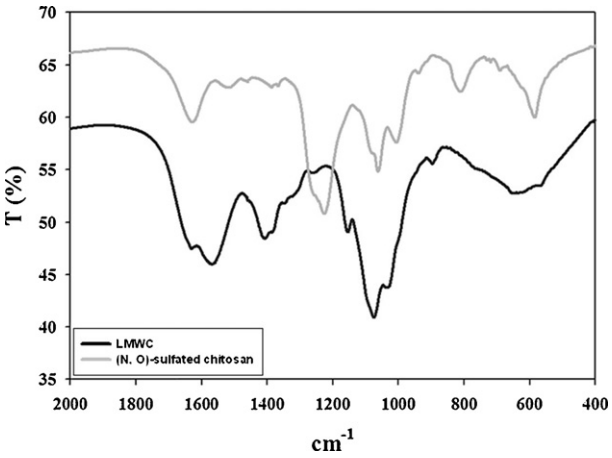

Fig. 1. FT-IR spectra of LMWC and (N,O)-sulfated chitosan.

3.2. Inhibition of adipocyte differentiation

In this study, we demonstrated that (N,O)-sulfated chitosan could negatively modulate 3T3-L1 adipocyte differentiation by regulating crucial adipogenic transcription factors and fat accumulation. The intracellular triglyceride accumulation is the major indicator for a full differentiation. Thus, inhibition of adipocyte differentiation was also exhibited by the means of decrease in intracellular triglyceride storages and increase in lypolysed triglycerides by the means of released glycerol content.

(N,O)-sulfated chitosan did not show any cytotoxicity up to concentration of 4 mg/ml on both non-differentiated preadipocytes and differentiated adipocytes (Table 1). 3T3-L1 preadipocytes completed their conversions to mature adipocytes in six days after stimulation to differentiate. However, treatment of the preadipocytes with (N,O)-sulfated chitosan starting from first day of differentiation decreased the cocktail-induced adipogenesis. Triglyceride accumulation of differentiated cells in the presence or absence of LMWC and (N,O)-sulfated chitosan at day 6 was checked by staining intracellular triglyceride droplets with Oil Red O. Images of stained cells (Fig. 2A) and eluted Oil Red O stain amounts (Fig. 2B) indicate LMWC and (N,O)-sulfated chitosan inhibited triglyceride accumulation of 3T3-L1 cells during adipogenic differentiation. (N,O)-sulfated chitosan-treated inhibition of the triglyceride accumulation occurred in a dose-dependent manner and the % inhibition at concentration of 1 mg/ml was 59.8 ± 4.6%, according to Oil Red O staining assay. Triglyceride-attached Oil Red O stain was evaluated as the relative percentage against untreated control adipocytes. Moreover (N,O)-sulfated chitosan suppressed the differentiation roughly two fold more than

Table 1
Cytotoxicity of (N,O)-sulfated chitosan on pre-adipocyte and adipocytes.

Cell line		
Concentration (mg/ml)	3T3-L1 Pre-adipocytes	3T3-L1 Adipocytes
0	100 ± 7.28[Aa]	100 ± 7.07[a]
0.1	97.39 ± 2.94[a]	102.18 ± 5.22[a]
0.25	99.29 ± 8.53[a]	105.08 ± 4.88[a]
0.5	97.94 ± 8.59[a]	97.19 ± 8.06[a]
1	90.07 ± 4.32[a]	97.15 ± 10.73[a]
2	98.86 ± 8.38[a]	101.52 ± 7.02[a]
4	91.10 ± 9.91[a]	98.14 ± 13.42[a]

Data is presented as percentage of untreated cells.
[a]Means with the different letters in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.
[A] Values represent means ± SD ($n = 3$).

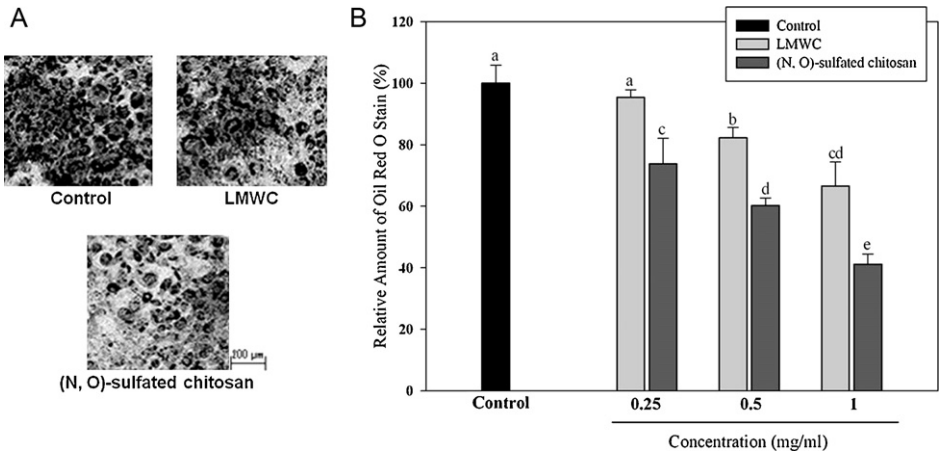

Fig. 2. Inhibitory effects of LMWC and (N,O)-sulfated chitosan on fat accumulation of 3T3-L1 cells after adipogenic differentiation. (A) Images of stained intracellular triglyceride storages. Images were taken just after the Oil Red O staining of the untreated control cells and cells treated with 1 mg/ml of LMWC and (N,O)-sulfated chitosan. The dark areas represent the lipid accumulation. (B) Levels of stained intracellular triglyceride according to eluted Oil Red O. [a–e] Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test. *Control*: fully differentiated control adipocytes (0.5 mM methyl-isobutylxanthine, 0.25 μM dexamethasone and 5 μg/ml insulin).

Table 2
Triglyceride contents (mg/mg protein) of LMWC and (N,O) sulfated chitosan treated adipocytes.

Concentration (mg/ml)				
Sample	0	0.25	0.5	1
LMWC	0.0945 ± 0.0036^{Aa}	0.0957 ± 0.0089^{ab}	0.0865 ± 0.0071^{ab}	0.0613 ± 0.0133^{bc}
(N,O)-sulfated chitosan	0.0945 ± 0.0036^{a}	0.0884 ± 0.0073^{ab}	0.0865 ± 0.0023^{c}	0.0375 ± 0.0062^{c}

[a–c] Means with the different letters in same row are significantly different ($p < 0.05$) by Duncan's multiple range test.
[A] Values represent means ± SD ($n = 3$).

that of LMWC, where the LMWC % inhibition of triglyceride accumulation was 31.5 ± 5.2%. Triglyceride amount in the differentiated cells was also measured with a commercial triglyceride assay kit to intensify the Oil Red O staining assay results. Intracellular triglyceride amount against per mg of the total protein amount of the cell was plotted to give more reliable results for inhibition of triglyceride accumulation (Table 2). Both LMWC and (N,O)-sulfated chitosan decreased the lipid amount in cells coordinately with staining results. Total triglyceride amounts after LMWC and (N,O)-sulfated chitosan treatments at the concentration of 1 mg/ml were 0.061 ± 0.01 and 0.038 ± 0.006 mg/mg protein, respectively, supporting the results of staining assay. Moreover, glycerol content of the cell culture medium at differentiation day 6 was calculated using a commercial free glycerol reagent. Released glycerol amount in the cell culture medium was raised from 41.9 ± 4.0 to 64.3 ± 3.7 and 83.2 ± 3.2 mg/ml after LMWC and (N,O)-sulfated chitosan treatment, respectively (Table 3). To assess the lipolytic response during adipocyte differentiation, the expression level of hormone-sensitive lipase (HSL) gene was determined by RT-PCR. Furthermore, expression levels of key adipogenic transcription factors and enzymes during differentiation were also evaluated with RT-PCR. Congruently, mRNA expression of several key adipogenic factors such as PPAR-γ, SREBP-1, C/EBP-α was decreased strongly by treatment of 1 mg/ml (N,O)-sulfated chitosan, while moderately decreased by 0.5 mg/ml (N,O)-sulfated chitosan (Fig. 3). Moreover, the presence of (N,O)-sulfated chitosan during adipocyte differentiation reduced HSL gene expression. However, under same conditions, important lipogenic regulator Acetyl coenzyme A carboxylase-1 (ACC-1) expression was slightly decreased without showing a significant change compared to adipogenic factor expressions. Concordantly, the mRNA expression levels were slightly decreased by treatment of 1 mg/ml LMWC while not changed by 0.5 mg/ml LMWC. In addition, effect of (N,O)-sulfated chitosan on adipogenic protein expression was tested by Western Blot analysis. (N,O)-sulfated chitosan also inhibited the mitogen-activated protein kinase (MAPK), PPAR-γ and C/EBP-α protein levels in a dose-dependent manner, compared to untreated cocktail-induced differentiated cells (Fig. 4).

Excessive lipid accumulation by adipocyte cells causes the advance of obesity. Moreover, lipid accumulation by differentiating cells is one of the key factors that represent adipogenic differentiation (Kawada, Takahashi, & Fushiki, 2001). Thus, inhibition of fat accumulation through triglycerides is one of the main ways to prevent onset and progression of obesity (McPherron & Lee, 2002). In this respect, the results which were presented through Oil Red O staining (Fig. 2) and triglyceride assay (Table 2), indicate a highly possible anti-obesity effect. LMWC and (N,O)-sulfated chitosan treatment resulted in the decrease of the intracellular

Table 3
Glycerol content (mg/ml) of culture medium of LMWC and (N,O)-sulfated chitosan treated adipocytes.

Concentration (mg/ml)				
Sample	0	0.25	0.5	1
LMWC	41.90 ± 4.02^{Aa}	42.02 ± 3.78^{a}	57.21 ± 6.24^{b}	64.78 ± 3.24^{b}
(N,O)-sulfated chitosan	41.90 ± 4.02^{a}	62.46 ± 5.66^{b}	78.71 ± 4.14^{c}	83.41 ± 2.98^{c}

[a–c] Means with the different letters in same row are significantly different ($p < 0.05$) by Duncan's multiple range test.
[A] Values represent means ± SD ($n = 3$).

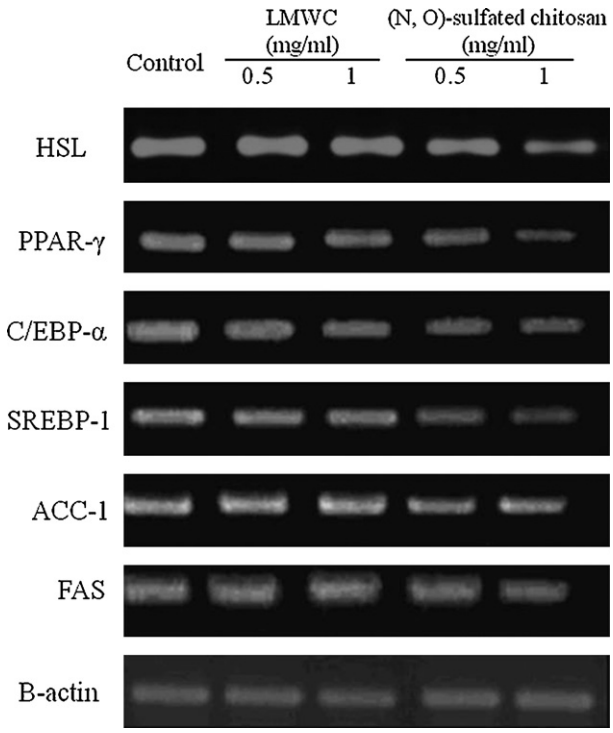

Fig. 3. mRNA expression levels of PPAR-γ, C/EBP-α, SREBP-1, FAS and ACC1 after LMWC and (N,O)-sulfated chitosan treatment during differentiation. β-Actin expression was used as an internal standard. *Control*: fully differentiated control adipocytes (0.5 mM methyl-isobutylxanthine, 0.25 μM dexamethasone and 5 μg/ml insulin).

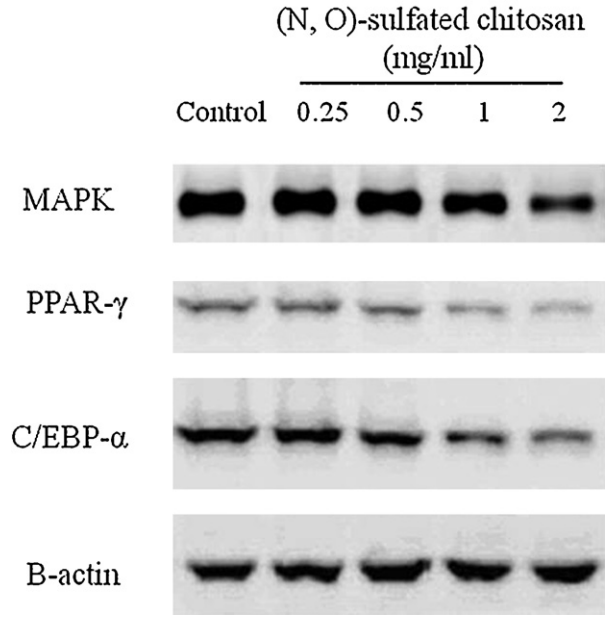

Fig. 4. Effect of LMWC and (N,O)-sulfated chitosan on protein levels of PPAR-γ and C/EBP-α from the total protein of differentiated cells at day 6 of differentiation. β-Actin was used as an internal standard. *Control*: fully differentiated control adipocytes (0.5 mM methyl-isobutylxanthine, 0.25 μM dexamethasone and 5 μg/ml insulin).

triglyceride amount. Furthermore, results of the glycerol assay (Table 3) suggested that, beyond the fat accumulation inhibitory effect, LMWC and (N,O)-sulfated chitosan treatment enhanced the triglyceride hydrolysis resulting in the decrease of the intracellular lipid amount.

Various transcription factors like PPAR-γ and C/EBP family are involved in Mitogen-activated protein kinase (MAPK) pathways during adipocyte differentiation. Among them, PPAR-γ, C/EBP-α and SREBP-1 have been elucidated to be positively involved in adipogenesis. Likewise, adipogenic differentiation also can be identified with lipogenic enzymes in fat metabolism such as FAS and ACC-1 (Kim & Spiegelman, 1996; Wu, Xie, Bucher, & Farmer, 1995). However, treatment of (N,O)-sulfated chitosan strongly inhibited PPAR-γ, C/EBP-α and SREBP-1 expression. However inhibition of FAS and ACC-1 was observed in a slight manner unlike the MAPK and related transcription factors, suggesting that (N,O)-sulfated chitosan regulation of adipocyte differentiation might occur through its regulation of the transcription factors via MAPK pathway rather than lipogenic enzymes. Moreover, Jae et al. (2008) reported that LMWC inhibits adipocyte differentiation affecting through the PPAR-γ cascade. In this regard, as stated in RT-PCR results (Fig. 3), both LMWC and (N,O)-sulfated chitosan inhibited the expression of key adipogenic factors such as PPAR-γ, C/EBP-α and SREBP-1. High protein levels of PPAR-γ and C/EBP-α regulated by elevated MAPK expression are also key markers for adipocyte differentiation pursuant to their elevated expression levels. In addition, as reported in Western blot analysis results (Fig. 4) (N,O)-sulfated chitosan decreased the MAPK, PPAR-γ and CEBP-α protein levels in a dose-dependent manner relative to untreated control cells. Furthermore, if these transcription factors are considered as the crucial mediators of adipocyte differentiation pathways, the (N,O)-sulfated chitosan-induced MAPK activation and inhibition of adipocyte differentiation can be partly occurred through down-regulation of these transcription factors by (N,O)-sulfated chitosan.

4. Conclusion

In the light of biochemical potential of sulfated chitosan derivatives, chitosan oligomers have been modified by sulfation and studied for their antiadipogenic activity. In conclusion, both LMWC and (N,O)-sulfated chitosan inhibited the adipogenic differentiation of 3T3-L1 cells, potentially through the PPAR-γ pathway as supported with RT-PCR and Western blot analysis results. (N,O)-sulfated chitosan could inhibit the mRNA expression of various adipocyte-specific factors such as PPAR-γ, C/EBP-α and SREBP-1. Correspondingly (N,O)-sulfated chitosan decreased the stored triglyceride levels and facilitated lypolysis demonstrated by released glycerol content. In addition, the expression levels of HSL gene was decreased by (N,O)-sulfated chitosan treatment. In the light of these results, it can be easily suggested that (N,O)-sulfated chitosan is more effective than LMWC with respect to inhibition of adipocyte differentiation. As a consequence, structural modification of LMWC by sulfation convincingly improved its anti-obesity effect in the matter of inhibiting adipogenic differentiation of 3T3-L1 cells.

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