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Chitosan Oligosaccharides Inhibit Adipogenesis in 3T3-L1 Adipocytes

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The 3T3-L1 cell line is a well-established and commonly used *in vitro* model to assess adipocyte differentiation. Over the course of several days, confluent 3T3-L1 cells can be converted to adipocytes in the presence of an adipogenic cocktail. In this study, the effects of chitosan oligosaccharides (CO) on adipocyte differentiation of 3T3-L1 cells were studied. The CO significantly decreased lipid accumulation, a marker of adipogenesis, in a dose-dependent manner. The low molecular mass CO (1–3 kDa) were the most effective at inhibiting adipocyte differentiation. Moreover, mRNA expression levels of both CCAAT/enhancer-binding protein (C/EBP) α and peroxisome proliferator-activated receptor (PPAR) γ, the key adipogenic transcription factors, were markedly decreased by CO treatments. CO also significantly downregulated adipogenic marker proteins such as leptin, adiponectin, and resistin. Our results suggest a role for CO as anti-obesity agents by inhibiting adipocyte differentiation mediated through the downregulated expression of adipogenic transcription factors and other specific genes.

Keywords: Adipocyte, chitosan oligosaccharide, obesity, 3T3-L1 cell, PPARγ, C/EBPα

It is known that adipocyte differentiation and the extent of subsequent fat accumulation are closely related to the occurrence and advancement of various diseases such as coronary artery disease and obesity [30, 36, 52, 57, 65].

3T3-L1 cells, originally derived from mouse embryos, have served as a useful *in vitro* model for adipocyte differentiation and function [14]. When stimulated to differentiate with an adipogenic cocktail containing dexamethasone, methylisobutylxanthine, fetal bovine serum, and insulin, these cultures take on adipocyte characteristics [38]. During differentiation of the adipocytes, a number of morphological and physiological changes occur. The 3T3-L1 cells change from spindle-shaped fibroblast cells to larger, spherical cells and accumulate large triglyceride droplets, which can be detected microscopically [27]. 3T3-L1 cells facilitate the investigation of regulatory mechanisms of adipocyte differentiation [18].

The differentiation of preadipocytes into adipocytes involves exposure of a confluent, quiescent population of cells to a variety of effectors that activate a cascade of transcription factors. This cascade begins with the CCAAT/enhancer-binding protein (C/EBP) β and C/EBPδ, which finally induce the expression of C/EBPα and peroxisome proliferator-activated receptor (PPAR) γ [41, 47]. These transcription factors coordinate the expression of genes involved in creating and maintaining the adipocyte phenotype, including the genes for adipocyte fatty acid binding protein, glucose transporter 4, lipoprotein lipase, and leptin [31, 62, 63].

Chitin and chitosan are polycationic polymers with a unique structure and specific properties [7]. Chitin is widely distributed in natural products such as the protective cuticles of crustaceans and insects, as well as in the cell walls of some fungi and microorganisms, and it is usually prepared from the shells of crabs and shrimps. Chitin is converted to chitosan by deacetylation with 45% NaOH at 100°C for 2 h. Chitosan oligosaccharides are obtained by enzymatic hydrolysis of chitosan [59]. Both chitosan and chitosan oligosaccharides (low molecular weight chitosan, CO) are commercially produced on a large scale and available in many countries [35, 55]. Several studies have shown that CO have anti-obesity effects [17, 21, 53, 59]. However, the effects of CO on the cellular and molecular mechanisms responsible for the differentiation and regulation of adipocytes gene expression have not been reported.

Therefore, the present study was undertaken to investigate the effects of CO on adipocyte differentiation in 3T3-L1 cells, with triglyceride accumulation being used as an indicator of differentiation. We also evaluated the mechanism of action of CO with respect to alterations

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in PPARγ and C/EBPα expression, as these are central regulators of adipocyte differentiation. In addition, modulation in the expression of important adipogenic proteins (*e.g.*, PPARγ, adiponectin, leptin, and resistin) was evaluated.

Materials and Methods

Reagents and Cells

3T3-L1 fibroblasts were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). Chitosan oligosaccharides were kindly provided from Kitto Life Co., Ltd. (Kyunggi, Korea). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Invitrogen Co., Carlsbad, CA, U.S.A.). Dexamethasone (DEX), 1-methyl-3-isobutyl xanthine (IBMX), and insulin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Culture and Differentiation of Adipocytes

Mouse 3T3-L1 preadipocytes were maintained in DMEM containing 10% FBS at 37°C in a humidified atmosphere of 5% CO_2. After 3T3-L1 cells reached confluency, the cells were incubated in differentiation medium (DM) containing 0.25 μM DEX, 0.5 mM IBMX, and 10 μg/ml insulin in DMEM with 10% FBS for 48 h. The cell culture medium was changed to post-DM containing 5 μg/ml insulin in DMEM with 10% FBS, and post-DM was freshly replaced every 48 h. Differentiated cells were only used when at least 95% of the cells showed an adipocyte phenotype by accumulation of lipid droplets. To determine the roles of CO in adipocyte differentiation, crude (containing a wide variety of molecular weights) and fractionated (containing three different ranges of molecular weights) CO were separately added to the medium at indicated concentration. The cells were harvested 8 days after the initiation of differentiation for RNA and triglyceride analysis, or Oil Red O staining, an indicator of cell lipid content.

Oil Red O Staining

The cells were washed with PBS twice, fixed with 10% formaldehyde at room temperature for 10 min, and stained with Oil Red O (Sigma) at 60°C for 10 min. Fat droplets in adipocytes were stained red. The pictures were taken using an Olympus (Tokyo, Japan) microscope (Fig. 1).

RT-PCR Analysis

Total RNA was isolated using RNAiso reagent (TAKARA, Otsu, Japan). RT-PCR was performed using the One Step RT-PCR kit

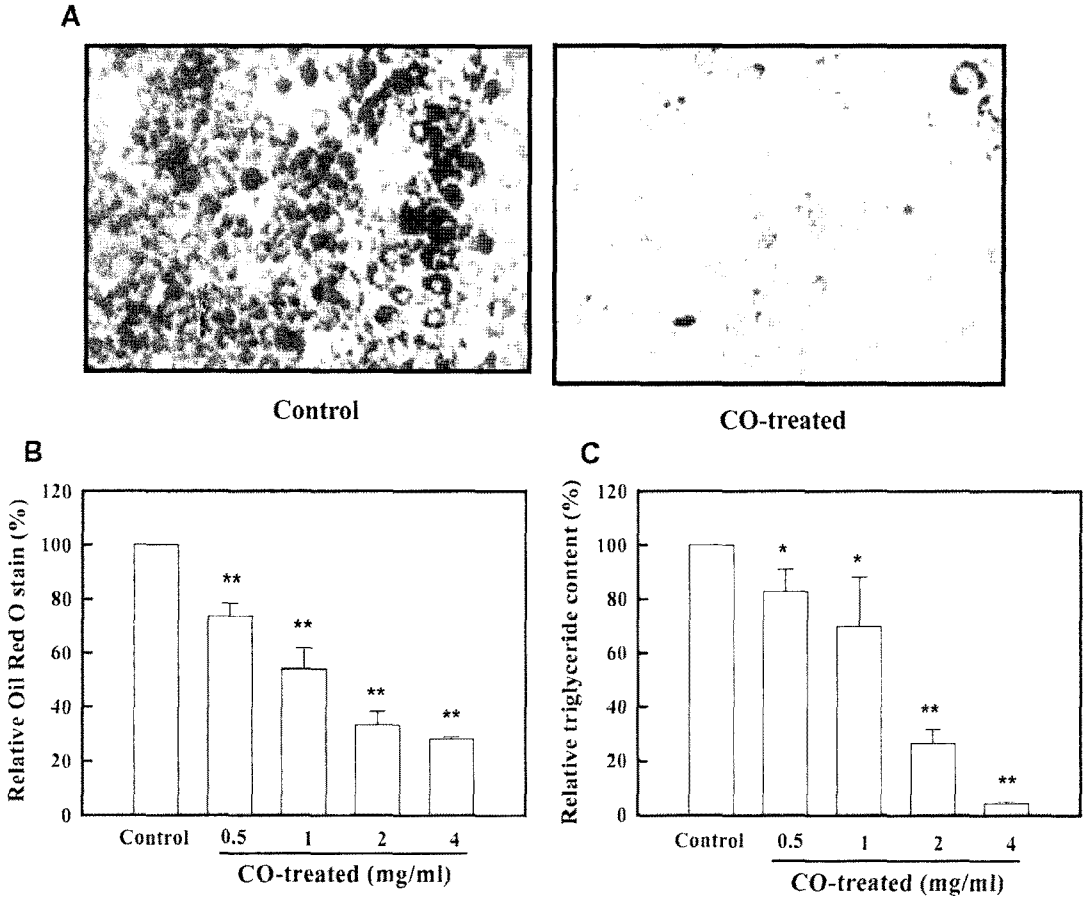

Fig. 1. Fat droplets in adipocytes in control and chitosan oligosaccharides (CO)-treated 3T3-L1 cells. **A**. Photograph of Oil Red O staining at day 5. **B**. and **C** Quantification of Oil Red O staining (**B**) and measurement of triglyceride content (**C**) at day 5.
Preadipocytes were cultured in growth medium until they reached confluency and quiescent cells were incubated in DM (differentiation medium) and post-DM with or without chitosan oligosaccharides for 8 days. *p<0.05 CO-treated vs. Control; ** p<0.001 CO-treated vs. control.

(Qiagen, Valencia, CA, U.S.A.) with gene-specific primers. The following primers were used:

PPAR-γ: forward 5'-GGTGAAACTCTGGGAGATTC-3'
reverse 5'-CAACCA-TTGGGTCAGCTCTT-3'
C/EBPα: forward 5'-AGGTGCTGGAGTTGACCAGT-3'
reverse 5'-CAGCCTAGAGATCCAGCGAC-3'
β-actin: forward: 5'-AGCCATGTACGTAGCCATCC-3'
reverse: 5'-CTCTCAGCTGTGGTGGTGAA-3'

PCR reactions were performed in a thermal cycler (Techne, Princeton, NJ, U.S.A.) under the following conditions: 50°C for 30 min, 95°C for 15 min (1 cycle); 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min (30 cycles). PCR products were electrophoresed on 1% agarose gel containing ethidium bromide (0.5 μg/ml) and were visualized as a single compact band of expected size under UV light and documented by a gel documentation system.

Triglyceride Assay

Treated 3T3-L1 adipocytes were washed gently with PBS and lysed with PBS supplemented with 0.1% NP40. Triglyceride content was measured using the Triglyceride GPO Trinder kit (Sigma). The absorbance was measured at 540 nm, and triglyceride was normalized to protein content determined by the Bradford assay [1].

Western Blot Analysis

The differentially regulated levels of the proteins of interest were confirmed by Western blot analysis as described below. Cells were homogenized and 10 μg of protein was diluted in 2× sample buffer [50 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol] and heated for five min at 95°C before SDS-PAGE (7.5 and 12%). Subsequently, they were transferred to a PVDF membrane (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and incubated overnight with 5% blocking reagent (Amersham Biosciences) in Tris-buffered salt (TBS) containing 0.1% Tween-20 at 4°C. The membrane was rinsed in four changes of TBS with Tween-20 (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 8.0), incubated twice for 5 min and twice for 10 min in fresh washing buffer, and then incubated for 2 h with blocking solution containing 1:200 dilution of primary antibody (rabbit anti-leptin and mouse anti-PPARγ, Santa Cruz Biotechnology; mouse anti-resistin, R&D Systems, Minneapolis, MN, U.S.A., and mouse anti-adiponectin, BioVision, Mountain View, CA, U.S.A.). After four washes, the membrane was incubated for 2 h in horseradish peroxidase-conjugated anti-goat IgG and anti-rabbit IgG secondary antibody (1:1,000, Santa Cruz Biotechnology) and developed using Enhanced Chemiluminescence (ECL Western blot analysis system kit; Amersham Biosciences). The Western blot was analyzed by scanning with a UMAX PowerLook 1120 (Maximum Technologies, Inc., Taipei, Taiwan) and digitalizing using an image analysis software (KODAK ID, Eastman Kodak Company, NY, U.S.A.).

Statistical Analysis

The results were analyzed for statistical significance by one-way analysis of variance (ANOVA) test using the Statistical Package of the Social Science (SPSS) program. All data were expressed as mean±SE ($P<0.05$). Group means were considered to be significantly different at $P<0.05$, as determined by the technique of protective least-significant difference (LSD).

Results

Inhibitory Effects of CO on 3T3-L1 Adipocyte Differentiation

To test whether CO inhibit adipocyte differentiation, we used insulin, dexamethasone, and isobutylmethyl xanthine (differentiation medium, DM) to induce 3T3-L1 preadipocyte differentiation. During the DM induction, CO were added to the medium at day 0 to observe their effects on 3T3-L1 adipocyte differentiation, and adipocytes were stained by Oil Red O. The staining results showed that 5-day incubation of crude CO (containing a wide variety of molecular mass CO) during the differentiation period significantly inhibited 3T3-L1 adipogenesis (see left panels of Fig. 1). It was found that treatment of 3T3-L1 cells with crude CO significantly decreased the cell differentiation (Fig. 1A) and triglyceride accumulation (Fig. 1B) in a dose-dependent manner, compared with control cells. Among the different molecular mass CO, the lower chain-length CO (1–3 kDa) was the most effective at reducing the triglyceride content (Fig. 2).

Effect of CO on Expression of Adipogenic Transcription Factors

To investigate the inhibitory mechanism of CO during the adipocyte differentiation, the expression of PPARγ and C/EBPα, key transcriptional factors for adipocyte differentiation, were examined. It is well documented that during DM induction of 3T3-L1 adipocyte differentiation, PPARγ and C/EBPs are activated by DM [45, 46]. For this aim, we purified total RNA from differentiated 3T3-L1 cells at day 5 and carried out RT-PCR. It was found that both PPARγ and C/EBPα were strongly inhibited (about 40%) by CO treatments at the transcriptional levels, suggesting that CO affect the signaling for adipocyte differentiation in 3T3-L1 (Fig. 3).

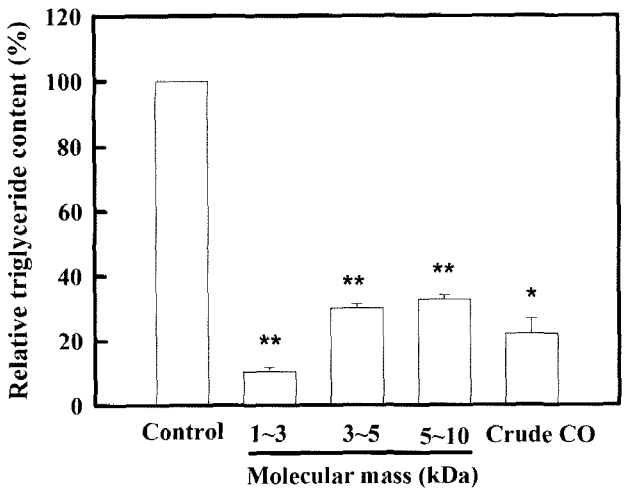

Fig. 2. Effect of chitosan oligosaccharides (CO) of different molecular masses on triglyceride content in 3T3-L1 cells.
*$p<0.05$ CO-treated vs. Control; ** $p<0.001$ CO-treated vs. Control.

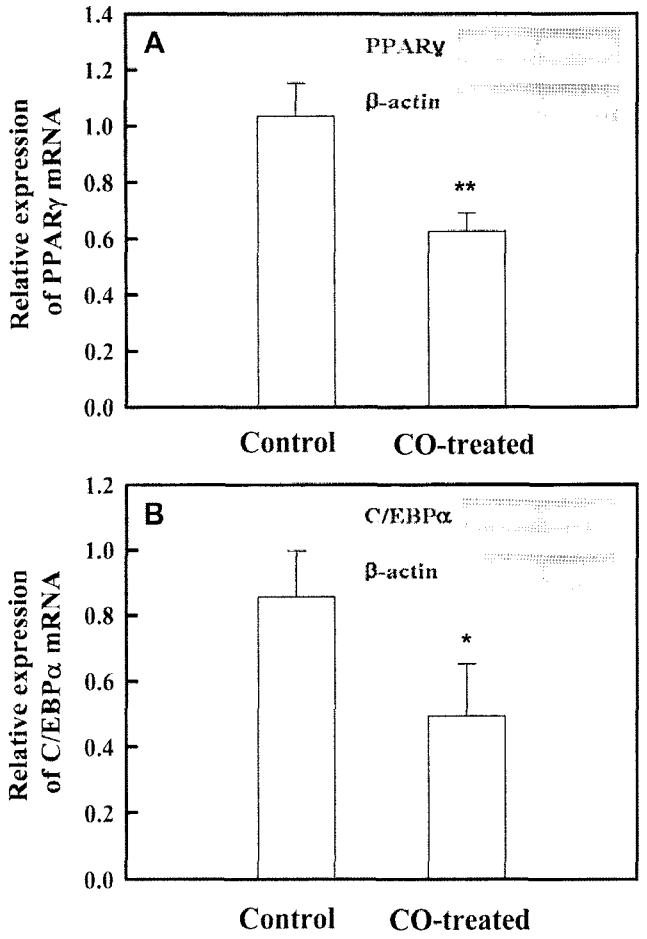

Fig. 3. Effect of chitosan oligosaccharides (CO) on mRNA expression of peroxisome proliferator-activated receptor γ (PPARγ) (panel **A**) and CCAAT/enhancer binding protein (C/EBP) α (panel **B**) analyzed by RT-PCR.
*$p<0.05$ CO-treated vs. Control; ** $p<0.001$ CO-treated vs. Control.

Effect of CO on Expressions of Adipogenic Marker Proteins

Since the PPARs mRNA and protein levels were inhibited by CO, we examined whether the target genes of PPARs were suppressed in 3T3-L1 adipocytes by CO treatment. Our Western blot analysis confirmed significant down-regulation of PPARγ, adiponectin, leptin, and resistin in protein levels (Fig. 4). In addition, fatty acid binding protein (FABP) and glucose transporter 4 (GLUT4), which are important biomarkers in adipogenesis, were significantly downregulated in response to CO treatment (Fig. 4). These results suggest that CO obviously inhibited lipogenesis and adipogenesis by downregulation of adipogenic marker proteins.

DISCUSSION

There is increasing interest in the use of natural resources as protective agents against obesity because chemically synthetic compounds have some harmful side-effects. For this reason, anti-obesity agents from natural products are being developed by many research groups [5, 10, 18, 22, 26, 33, 38, 44]. As for chitin/chitosan-derived biomaterials, conflicting reports have recently been documented about their anti-obesity effects. For example, Gallaher *et al.* [13] demonstrated that chitosan and CO decreased plasma cholesterol and increased cholesterol excretion in overweight normocholesterolemic humans. Other investigators also indicated positive roles of chitosan for hypocholesterolemic effects in mice [17, 59]. In contrast, there are different results claiming that the anti-obesity effects of those biomaterials are clinically negligible in humans [11, 12, 16, 20].

At the molecular level, the adipocyte differentiation process is regulated by transcriptional activators such as C/EBPα, PPARγ, and CBP/p300, and some transcriptional repressors [15, 37, 61].

PPARγ is a member of the nuclear receptor superfamily of transcription factors and is predominantly expressed in adipose tissue. These transcription factors appear to function as dominant activators of adipocyte differentiation [3]. Overexpression of PPARγ induces adipocyte differentiation in 3T3-L1 adipocytes. On the other hand, suppression of PPARγ expression blocks adipogenesis and lipogenesis. PPARs mediate the transcription of a group of genes related to fatty acid synthesis, oxidation, transport, storage, or energy expenditure [26, 56].

C/EBP (C/EBPα, β, and δ) belongs to the basic leucine zipper family of transcription factors [26, 69]. It was reported that C/EBPα is a likely candidate transcription factor for directly regulating adipocyte differentiation [69]. C/EBPα expression was highest in the extraperitoneal adipose tissue of obese subjects, which is known to harbor larger and more fat-laden adipocytes [6, 43]. Hence, increased C/EBPα synthesis may be required for maintaining adipocyte function when fat cell size increases and obesity advances.

C/EBPα and PPARγ interchangeably bind to their genomic promoter regions to activate transcription and maintain their own activity [30]. In the present study, we showed that CO treatment significantly decreased both C/EBPα and PPARγ mRNA expression in cultured 3T3-L1 adipocytes (Figs. 3A and 3B) and inhibited protein expression of PPARγ (Fig. 4). These combined observations suggest that CO suppress adipocyte differentiation through C/EBPα- and PPARγ-mediated adipogenesis mechanisms.

Several marker proteins in adipocyte differentiation have been well documented. For example, leptin is exclusively secreted by adipocytes in proportion to their triglyceride stores, and circulating leptin levels are correlated with the extent of obesity [19, 40]. It regulates energy expenditure and food intake through the central nervous system. Obese subjects have higher leptin levels in comparison with normal-weight or lean subjects. Therefore, leptin is used as an indicator of obesity *in vivo* and *in vitro*. In this study,

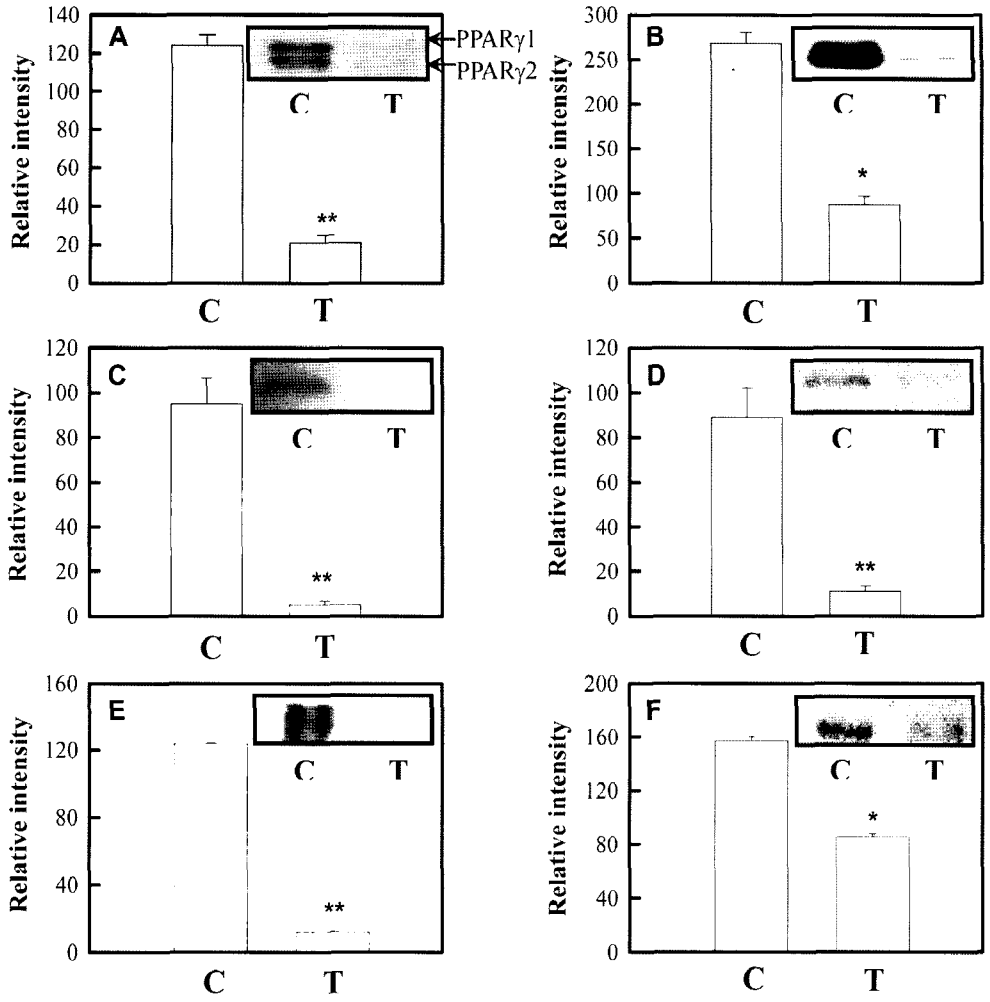

Fig. 4. Effect of chitosan oligosaccharides (CO) on protein expressions of (**A**) PPARγ, (**B**) adiponectin, (**C**) leptin, (**D**) resistin, (**E**) FABP, and (**F**) GLUT4 in 3T3-L1 cells by Western blot analysis.
C: control cells; T: CO-treated cells. Band density was digitized with software and mean±SE of three independent experiments are shown; *$p<0.05$ CO-treated vs. Control; ** $p<0.001$ CO-treated vs. Control.

CO treatment significantly decreased leptin secretion, indicating that it decreased lipid accumulation (Fig. 4).

Adiponectin is also exclusively expressed in adipocytes [23, 49]. Adiponectin gene expression is turned on 2 days after the initiation of adipocyte differentiation and maintained at a relatively high level in mature adipocytes. Blockage of adipocyte differentiation reduces adiponectin expression.

Resistin is a member of the newly discovered family of cysteine-rich secretory proteins [58]. To date, studies of resistin expression in adipocytes differentiation have been controversial. For example, secretion of resistin has been shown to increase during adipogenesis of the 3T3-L1 murine clonal cell line and of primary cultured stromal cells derived from rat adipose tissues [25, 34]. In contrast, it was found that the expression of resistin mRNA and protein was found to be strongly suppressed in adipose tissues in many models of experimental obesity [8, 9, 66]. However, Janke *et al.* [25] reported no relationship between insulin resistance and the expression of the resistin gene in human subcutaneous abdominal adipocytes. Their findings in humans seem to contradict previous reports in mice and murine colonal cell lines. In our investigation, expectedly, CO suppressed gene expressions of adiponectin and resistin in 3T3-L1 adipocytes.

GLUT4 and FABP mediate insulin-stimulated glucose uptake and serve as the critical link between lipid metabolism, hormone action, and cellular functions in adipocytes [29, 39]. Adipose tissue acts as an additional storage site and plays a critical role in maintaining insulin responsiveness, glucose homeostasis, and lipid metabolism [2, 48, 54, 67]. We demonstrated a marked reduction in GLUT4 and FABP expression levels with CO treatment. This may be mediated by three classes of transcription factors, namely peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding proteins (C/EBPs), and sterol regulatory element binding protein 1c (SREBP-1c) [47], which are well known for their direct influence on adipogenesis [28, 50]. C/EBPβ/δ triggers the transcriptional activation of PPARγ and C/EBPα [24, 42] through a

combination of cross- and auto-regulation. These two key regulators of adipogenesis mediate the acquisition of adipocyte phenotype [4, 28, 37]. PPARγ target genes in adipose tissue are directly implicated in lipogenic pathways (*e.g.*, adipocyte fatty acid binding protein (FABP) and GPDH activity [32]), and also C/EBPα was found to bind to the promoters of FABP [51] and GLUT4 [68]. SREBP-1c is highly expressed in adipose tissue and liver, and is expressed during early adipocyte differentiation [32, 64], which stimulates the expression of several lipogenic genes [32, 60]. Thus, C/EBPα, PPARγ, and SREBP-1c are part of the adipocyte differentiation program, which induces the maturation of preadipocytes into lipid-containing fat cells.

Taken together, these results indicate that suppression of adipocyte differentiation by CO treatment may be mediated, in part, through downregulation of the adipogenic transcription factors and other specific genes. To the best of our knowledge, this is the first report to demonstrate the anti-obesity effects of CO on the basis of molecular mechanism. Thus, CO may prove to be valuable natural products in the treatment of obesity, albeit *in vivo* studies are necessary to further explore this hypothesis.

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