Long-chain bases from *Cucumaria frondosa* inhibit adipogenesis and regulate lipid metabolism in 3T3-L1 adipocytes

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Abstract This study aims to investigate anti-adipogenic effects of long-chain bases from *Cucumaria frondosa* (Cf-LCBs) *in vitro*. Results showed that Cf-LCBs inhibited adipocyte differentiation and the expressions of CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferators-activated receptor γ (PPAR γ). Cf-LCBs increased β -catenin mRNA and nuclear translocation and increased its target genes, cyclin D1 and c-myc. Cf-LCBs enhanced fizzled and lipoprotein-receptor-related protein5/6 (LRP5/6) expressions, whereas wingless-type MMTV integration site10b (WNT10b) and glycogen synthases kinase 3 β (GSK3 β) remained unchanged. Cf-LCBs also reduced adipogenesis and recovered WNT/ β -catenin signaling in the cells suffering from 21H7, a β -catenin inhibitor. In addition, Cf-LCBs decreased triglyceride content and the expressions of lipogenesis genes. Cf-LCBs increased FFA levels and the expressions of lipolytic factors. Cf-LCBs promoted the phosphorylation of adenosine-monophosphate-activated protein kinase (AMPK) and acetyl-CoA carboxylase. These findings indicate that Cf-LCBs inhibit adipogenesis through activation of WNT/ β -catenin signaling and regulate lipid metabolism *via* activation of AMPK pathway.

Keywords: *Cusumaria frondosa*, long-chain bases, adipocyte differentiation, lipid metabolism

Introduction

Obesity has been considered as a serious health problem worldwide, which is positively associated with the occurrence of hyperlipidaemia, insulin resistance, type-2 diabetes, and other metabolic syndromes (1). Increased adipose tissue mass is known to directly result in obesity. Adipose tissue plays an important role in energy balance and changes in mass according to the metabolic requirements of the organism (2). Both the number and size of adipocytes in individuals can result in the increase in fat mass, which in turn depends on differentiation from preadipocytes (3). Mature adipocyte differentiation is mainly controlled by two key families of transcriptional factors, CCAAT/enhancer binding proteins (C/EBPs) and peroxisome-proliferators-activated receptor γ (PPAR γ) (4). C/EBP α and PPAR γ are required for adipocyte differentiation because they are involved in the growth arrest (5). Meanwhile, the two adipogenic transcription factors are controlled by a series of signaling cascades, in which wingless-type MMTV integration site (WNT)/ β -catenin signaling is the most important (6). WNT/ β -catenin signaling has been shown to negatively regulate adipogenesis (7). In this signaling, Wnt10b binds to the receptors, including fizzled (FZL) and low-density lipoprotein-receptor-related proteins (LRPs), which finally result in β -catenin

nuclear translocation (8). Nuclear β -catenin in turn mediates the activation of WNT response proteins, such as cyclin D1 (CCND1) and c-myc, and subsequently suppresses C/EBP α and PPAR γ expressions, causing a further decrease in adipogenesis (9). In contrast, during the terminal phase of differentiation, the activation of the transcriptional cascade results in an increase or decrease of activity of proteins and enzymes involved in the synthesis and degradation of triacylglycerol and fatty acids, such as glycerol-3-phosphate acyltransferase (GPAT), fatty acid synthase (FAS), hormone sensitive lipase (HSL), and carnitine palmitoyl transferase-1 (CPT-1) (10). These proteins or enzymes are regulated by adenosine-monophosphate-activated protein kinase (AMPK) (11). Activating AMPK signaling can reduce the synthesis of fatty acids and triacylglycerols, and increase fatty acids oxidation and triacylglycerols decomposition (12).

Long-chain bases (LCBs), also called sphingoid bases, are the simplest members in the glycosphingolipids family. Current studies show that LCBs possess several bioactivities, such as anti-oxidation, anti-inflammatory responses, antitumor, improvement in type-2 diabetes, and inhibition of keratinocyte differentiation (13–17). LCBs from marine organisms exhibit better bioactivities because of their special environment (18). Therefore, increasing attentions have been paid to LCBs extracted from sea cucumbers (SC-LCBs), the popular

Fig. 1. Main chemical structure of Cf-LCBs.

traditional seafood in China. SC-LCBs were reported to induce apoptosis in human hepatoma HepG2 cells through p-AKT and DR5 (19). SC-LCBs also ameliorated glucose tolerance and hepatic triglyceride (TG) content in obese mice (20). However, the effects of SC-LCBs on adipogenesis and lipid metabolism are still unknown. Therefore, we first investigated their effects on adipogenesis and lipid metabolism *in vitro* and clarified their underlying mechanism.

Materials and Methods

Preparation of Cf-LCBs Dried *Cucumaria frondosa* used in the study was purchased from Nanshan aquatic products market (Qingdao, China). Cf-LCBs were extracted and analyzed as previously described (21). The yield was approximately 1.47%. Their molecular weights were in the range of 238.4–320.5 Da, analyzed by the ESI-MS method. The main chemical structures of Cf-LCBs are shown in Fig. 1.

Cell lines, cell culture, and adipocyte differentiation Mouse 3T3-L1 fibroblast cells were obtained from ATCC (Manassas, VA, USA) and cultured in dulbecco's modified eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 U/mL penicillin at 37°C in a humidified atmosphere of 5% CO₂. The fibroblast cells were incubated in 24 well plates for 48 h until confluence. At this point (day 0), the cells were exposed to standard differentiation inducers (DMEM with 10% FBS, 10 mg/L insulin, 0.5 mM isobutylmethylxanthine, and 1 µM dexamethasone) for 48 h (from day 0 to day 2). This treatment was repeated once (from day 2 to day 4). Differentiated adipocytes were used in experiments at day 8 of differentiation.

The viability and cytotoxicity of 3T3-L1 cells To measure the viability and cytotoxicity during the differentiation process, mature adipocytes were cultured with different concentration of Cf-LCBs (0, 25, 50, and 100 µg/mL) from day 0 in 96 well plates. Cell viability was assayed at day 4 by the MTT method. Cell cytotoxicity was assessed through the activity of lactate dehydrogenase (LDH) in the culture

supernatant of 3T3-L1 cells at day 4.

Oil-red O staining The differentiating mature adipocytes were cultured with different concentrations of Cf-LCBs (0, 25, 50, and 100 µg/mL) from day 0, day 2, or day 4. At day 8, 3T3-L1 cells were washed twice with PBS, fixed through incubation with 10% fresh formaldehyde in PBS for 1 h, washed with PBS three times, and then stained with filtered 0.5% Oil-red O (Sigma-Aldrich, St. Louis, MO, USA) solution for 0.5 h. The stained lipid droplets were photographed with an inverted microscope (IX51; Olympus, Tokyo, Japan) and dissolved in isopropanol, and quantified by measuring the absorbance value at 570 nm.

Triglyceride (TG) and free fatty acids (FFAs) concentration analysis

Mature adipocytes were cultured for 24 h with different concentrations of Cf-LCBs. The culture medium was collected to measure FFA contents using an ELISA kit (Invitrogen, Carlsbad, CA, USA), and the cells were lysed to test intracellular TG content using a commercial TG assay kit (Biosino, Beijing, China). TG level is expressed as TG content/intracellular protein content (mg/mg protein).

Quantitative real-time PCR (qRT-PCR) analysis

Adipocyte-differentiation-related gene mRNA expressions were examined by qRT-PCR, including WNT10b, FZL, LRP5, LRP6, glycogen synthases kinase 3β (GSK3β), β-catenin, CCND1, c-myc, PPARγ, and C/EBPα. 100 µg/mL Cf-LCBs and 50 µM 21H7 (Sigma-Aldrich) were used at day 0. Unless otherwise specified, 100 µg/mL of Cf-LCBs were used throughout the experiments. The RNA was extracted from the cells with TRIzol reagent according to the manufacturer's instructions (Invitrogen) at day 0, day 2, day 4, and day 8, and cDNA was synthesized from 1 µg of RNA using a PCR thermocycler (iQ5; Bio-Rad, Hercules, CA, USA). PCR was tested by amplification of 15 ng cDNA in a 25 µL reaction containing SYBR-Green mix using iQ5 Real-Time PCR System. Cycling conditions were at 95°C for 10 min, at 95°C for 15 s, at 60°C for 20 s, and at 72°C for 30 s with 45 cycles. The primers were listed in Table 1. Data were analyzed using the software of iCycler iQ5. The level of expression of the target gene was normalized to that of β-actin.

Table 1. PCR primers used in the genes mRNA expression research

Genes	Forward primer	Reverse primer
WNT10b	5'-ATGCGGATCCACAACAACAG-3'	3'-CTTCACGTTTACGGTACCTT-5'
FZl	5'-CAGCAGTACAACGGCGAAC-3'	3'-CGGTGTGCTTAGTCCTCTG-5'
LRP5	5'-ACCGGCTGGACAAGTTCATC-3'	3'-GGTTTCGGACTCGGGTCT-5'
LRP6	5'-ACCTCAATGCGATTGTTC-3'	3'-CGTCTCCGAAGAACTGTGG-5'
GSK3 β	5'-ACCCTCATTACCTGACCTT-3'	3'-TCGGCAGACAATTCAACTC-5'
β -Catenin	5'-GATTTCAGGTGACGAGGA-3'	3'-TGTTGAACGGTTCGTGTAC-5'
CCND1	5'-GCGTACCCTGACACCAAT-3'	3'-TTCACGCACGCTTCTCTA-5'
c-myc	5'-TGATGACCGAGTTACTTGAG-3'	3'-CGTTTCTGTCGTGGTGG-5'
PPAR γ	5'-CGCTGATGCACTGCCTATGA-3'	3'-CCTTAGTCGAGACACCTGGAGA-5'
C/EBP α	5'-GAACAGCAACGAGTACCGGTA-3'	3'-GAGGAACGAGTCCGGTACCG-5'
β -Actin	5'-TGTCCACCTTCCAGCAGATGT-3'	3'-AGATCCGCTGACAATGACTCGA-5'

Separation of nuclear fraction To obtain the nuclear protein, adipocytes (treated with 100 μ g/mL Cf-LCBs) at day 4 were digested with trypsin, and nuclear fraction was then obtained using a nuclear extracts kit (Solarbio, Beijing, China). The obtained nuclear protein was used for western blotting.

Western blot assay 3T3-L1 cells (treated with 100 μ g/mL Cf-LCBs) at day 4 were lysed in IP lysis buffer to dissolve cellular protein. Equal amount of proteins or 100 μ g nuclear proteins were resolved by 10% polyacrylamide gels containing sodium dodecyl sulfate and transferred to polyvinylidene fluoride membranes. The membranes were blotted through incubation for 2 h with 5% BSA. Protein was incubated overnight at 4°C with primary antibody (PPAR γ , C/EBP α , β -catenin, GPAT, HSL, CPT-1, p-AMPK, and AMPK were acquired from Abcam, Cambridge, MA, USA; LRP6, FAS, p-ACC, ACC, and β -actin were acquired from Cell Signaling, Boston, MA, USA) and subsequently incubated for 2 h with horseradish-peroxidase-conjugated secondary antibody. Immunodetection was performed using ECL. β -Actin was used as control for protein expression and the corresponding total protein for the phosphorylated protein.

Statistical analysis Data are expressed as the mean $\pm$ standard deviation (SD). A one-way analysis of variance (ANOVA), followed by Tukey's test or Student's *t*-test with SPSS version 17.0 software, was used for multiple comparisons. Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Cf-LCBs inhibit 3T3-L1 cells differentiation Suppression of lipogenesis and adipogenesis is a feasible approach against obesity. SC-LCBs can reduce hepatic TG content and body weight gain in obese mice (20); however, the approach to act on adipocytes is still not understood. As shown in Fig. 2A, 50 and 100 μ g/mL Cf-LCBs significantly reduced the relative activity of 3T3-L1 cells ($p < 0.01$), suggesting that Cf-LCBs can inhibit adipocyte proliferation. Cf-LCBs could not influence the LDH activity in the culture supernatant (Fig. 2B), which suggested

that Cf-LCBs had no cytotoxicity on 3T3-L1 cells. The results of Oil-red O staining and Oil-red O absorbance (Fig. 2C and 2D) showed that the number of lipid droplets were all lower than those in the Cf-LCBs-treated groups from day 0 and day 2 in their corresponding control groups. Moreover, 100 μ g/mL Cf-LCBs-treated group from day 4 significantly reduced the number of lipid droplets compared to the control group ($p < 0.05$). These findings suggest that Cf-LCBs can inhibit adipocyte differentiation, indicating the potent anti-obesity activity of Cf-LCBs.

Adipocyte differentiation is associated with lipogenesis, which is regulated through several transcriptional factors. PPAR γ and C/EBP α are the crucial and indispensable transcriptional factors in the final phase of adipocyte differentiation and are linked to obesity and metabolic diseases. No one receptor can induce adipocyte differentiation, except PPAR γ (22). Figure 2E and 2G showed that the mRNA expressions of PPAR γ and C/EBP α were significantly reduced in the cells treated with 100 μ g/mL Cf-LCBs at day 4 and day 8 compared with the control ($p < 0.01$). Further studies using western blotting showed that Cf-LCBs inhibited the protein expressions of PPAR γ and C/EBP α at day 4 ($p < 0.01$, Fig. 2F and 2H). Several studies proved that adipocyte differentiation was suppressed when PPAR γ and C/EBP α protein levels were reduced; however, PPAR γ and C/EBP α overexpressions could cooperate in activation of the adipocyte gene program (23-26). In the present study, Oil-red O staining showed the inhibition of adipogenesis by Cf-LCBs, associated with the down-regulation of PPAR γ and C/EBP α at transcriptional and translational levels. These indicate that Cf-LCBs can significantly suppress adipocyte differentiation.

Cf-LCBs activate the WNT/ β -catenin pathway WNT/ β -catenin signaling has been proven to be one of the pivotal pathways to inhibit adipocyte differentiation. The activated WNT/ β -catenin pathway is adverse to adipocyte differentiation through the transfer of β -catenin protein into the nucleus. Canonical WNT/ β -catenin pathway is transduced through FZl family receptors and the LRP5/LRP6 co-receptor to the β -catenin signaling cascade (7). β -Catenin is the pivotal point of the canonical WNT/ β -catenin pathway. High-level translocation into the nucleus of β -catenin could prevent the

Fig. 2. Effects of Cf-LCBs on adipocytes proliferation and differentiation. 3T3-L1 cells were induced to mature adipocytes and stained with oil-red O at day 8. The mRNA expression was measured by qRT-PCR and protein expression by Western blotting. A, adipocyte relative activity; B, LDH activity; C, Oil-red O stain; D, Oil-red O absorbance; E, PPARγ mRNA; F, PPARγ protein; G, C/EBPα mRNA; H, C/EBPα protein. Data are expressed as mean±SD. Multiple comparisons were performed using Tukey's test for A, B, and D; using Student's *t*-test for E, F, G, and H. **p*<0.05; ***p*<0.01 versus control.

adipogenesis through activating the WNT/β-catenin signaling cascade (8,9). When β-catenin translocates into the nucleus, its target genes are elevated, including CCND1 and c-myc (6). These target genes then inhibit adipogenesis by down-regulating PPARγ and C/EBPα (27). In contrast, PPARγ can counteract the canonical WNT/β-catenin pathway during adipocyte differentiation (7). WNT10b binding to FZL and LRP transmembrane receptors initiates the canonical WNT/β-catenin signaling (28). WNT10b maintains preadipocytes in an undifferentiated state through the WNT/β-catenin pathway and by

repressing adipogenesis (29). The interactions between canonical WNTs and their receptors induce the stabilized β-catenin entering the nucleus and promote the transcription of WNT target genes, such as CCND1 and m-myc (30). Cho *et al.* (31) reported that the down-regulation of WNT10b expression could enhance adipogenesis in 3T3-L1 cells. Xu *et al.* (32) reported that fucosylated chondroitin sulfate from sea cucumber exhibited anti-adipogenic activity by enhancing WNT10b, FZL, and LRP. To investigate the effects of Cf-LCBs on WNT/β-catenin signaling, mRNA levels of the key regulators of

Fig. 3. Effects of Cf-LCBs on WNT/β-catenin pathway. The mRNA expression was measured by qRT-PCR and protein expression by western blotting. A, WNT10b mRNA; B, LRP5 mRNA; C, LRP6 mRNA; D, FZL mRNA; E, GSK3β mRNA; F, β-catenin mRNA; G, CCND1 mRNA; H, c-myc mRNA; I, LRP6 protein; J, total β-catenin protein; K, nuclear β-catenin protein. Data are expressed as mean±SD. Multiple comparisons were performed using Student's *t*-test. **p*<0.05; ***p*<0.01 versus control.

this pathway, namely WNT10b, FZL, LRP5, LRP6, β-catenin, GSK3β, CCND1, and c-myc, were detected. q-RT-PCR results showed that Cf-LCBs did not change the WNT10b mRNA expressions (Fig. 3A). Cf-LCBs significantly increased mRNA expressions of the upstream members in the pathway, including FZL, LRP5, LRP6, and β-catenin ($p<0.05$, $p<0.01$) (Fig. 3B, 3C, 3D, and 3F). However, there was no change in GSK3β mRNA expressions (Fig. 3E). The downstream gene mRNA expressions, CCND1 and c-myc, were also increased in Cf-LCBs-treated cells ($p<0.05$, $p<0.01$) (Fig. 3G and 3H). Figure 3I suggests that Cf-LCBs significantly increased LRP6 protein expression ($p<0.01$). Moreover, Cf-LCBs elevated total and nuclear β-catenin

(Fig. 3J and 3K), suggesting the increased β-catenin nuclear translocation by stimulation of Cf-LCBs. These results indicate that Cf-LCBs inhibit adipocyte differentiation by activating WNT/β-catenin signaling.

To further evaluate the role of Cf-LCBs-activated WNT/β-catenin pathway on adipocyte differentiation, 21H7, a β-catenin inhibitor, was added into the cells. β-Catenin mRNA expression was not changed between the control and 21H7-suffered cells (Fig. 4A). However, 21H7 significantly decreased the total β-catenin protein expression ($p<0.05$) and nuclear β-catenin level ($p<0.01$) (Fig. 4B and 4C). Cf-LCBs significantly increased β-catenin mRNA level, nuclear β-

Fig. 4. Effects of Cf-LCBs on adipocytes differentiation under 21H7. 3T3-L1 cells were induced to mature adipocytes: incubated with 50 μ M 21H7: and stained with Oil-red O at day 8. The mRNA expression was measured by qRT-PCR and protein expression by western blotting. A, β -catenin mRNA; B, total β -catenin protein; C, nuclear β -catenin protein; D, CCND1 mRNA; E, m-myc mRNA; F, Oil-red O stain; G, Oil-red O absorbance; H, PPAR γ mRNA; I, PPAR γ protein; J, C/EBP α mRNA; K, C/EBP α protein. Data are expressed as mean $\pm$ SD. Multiple comparisons were performed using one-way ANOVA analysis followed by Tukey's test. * p <0.05; ** p <0.01 versus control; # p <0.05; ## p <0.01 versus 21H7.

catenin protein, CCND1, and c-myc mRNA (Fig. 4B, 4C, 4D, and 4E). Meanwhile, 21H7 also retarded total β -catenin protein by 45.4%, although there is no obvious significance. Figure 4F and 4G showed that Cf-LCBs suppressed 21H7-elevated lipid accumulation. Cf-LCBs

also significantly increased the mRNA and protein expressions of C/EBP α and PPAR γ in 21H7-suffered cells (Fig. 4H, 4I, 4J, and 4K). In this experiment, the results further prove that Cf-LCBs can reduce adipogenesis through WNT/ β -catenin signaling.

Fig. 5. Effects of *Cf*-LCBs on lipid metabolism and AMPK pathway in adipocytes. 3T3-L1 cells were induced to mature adipocytes and incubate with different concentrations of *Cf*-LCBs for 24 h. TG and FFAs contents were measured using the TG kit and FFAs ELISA kit. The protein expression was measured by western blotting. A, TG contents; B, FFAs levels; C, GPAT protein; D, FAS protein; E, HSL protein; F, CPT-1 protein; G, phosphorylated AMPK protein; H, phosphorylated ACC protein. Data are expressed as mean $\pm$ SD. Multiple comparisons were performed using one-way ANOVA analysis followed by Tukey's test. * p <0.05; ** p <0.01 versus control.

Cf-LCBs regulate lipid metabolism of 3T3-L1 cells The above experiments showed that *Cf*-LCBs reduced lipid accumulation. We next studied the effects of *Cf*-LCBs on lipid metabolism. Figure 5A showed that 50 and 100 µg/mL *Cf*-LCBs could significantly reduce TG content (p <0.05, p <0.01). Meanwhile, the FFA levels in the culture supernatant were significantly increased in 25, 50, and 100 µg/mL *Cf*-LCBs-treated cells (Fig. 5B), which suggested that *Cf*-LCBs might promote lipolysis. The protein expression of the key gene related to lipogenesis and lipolysis was measured to further explore how *Cf*-LCBs regulate lipid metabolism. As shown in Fig. 5C and 5D, 25, 50, and 100 µg/mL *Cf*-LCBs significantly reduced GPAT and FAS protein expressions, the pivotal lipogenesis related factors. The lipolytic genes, HSL and CPT-1, were markedly increased by *Cf*-LCBs (50 and 100 µg/mL) in 3T3-L1 cells (Fig. 5E and 5F). Thus, *Cf*-LCBs can reduce lipid accumulation in mature adipocytes by inhibiting the lipogenesis process and promoting the lipolysis process.

Cf-LCBs activate AMPK signaling AMPK has been suggested to function as a sensor of cellular energy status, and the phosphory-

lated AMPK can inhibit the activity of ACC through phosphorylating ACC, the rate-limiting enzyme in fatty acid biosynthesis, and then decelerate lipogenesis in 3T3-L1 cells (33). Meanwhile, activated AMPK can down-regulate the expressions of other fatty acid biosynthesis-related enzymes, such as FAS and GPAT (34). In contrast, when AMPK is phosphorylated, the expressions of HSL and CPT-1 are up-regulated to accelerate lipolysis (35). As *Cf*-LCBs regulated lipid metabolism, we next surveyed the effects of *Cf*-LCBs on the AMPK pathway. As shown in Fig. 5G and 5H, *Cf*-LCBs significantly increased the phosphorylated protein expression of AMPK and ACC in mature adipocytes (p <0.05, p <0.01). These results suggest that *Cf*-LCBs can regulate lipid metabolism *via* activation of AMPK signaling.

In summary, this study proved that *Cf*-LCBs were capable of inhibition in adipogenesis in 3T3-L1 cells. Activation of WNT/ β -catenin pathway was the underlying mechanism for *Cf*-LCBs-mitigated adipogenesis, in which elevation of WNT10b, FZL, and LRP6, nuclear translocation of β -catenin, and increases in target factors of β -catenin, were involved. Moreover, *Cf*-LCBs promoted lipid metabolism through enhancing phosphorylation of AMPK and ACC.

It provides significant implication for the application of LCBs as functional foods inhibiting obesity.

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