Food Sci. Biotechnol. Vol. 16, No. 2, pp. 212 ~ 217 (2007)

Food Science and Biotechnology

Effect of Fucoidan on Expression of Diabetes Mellitus Related Genes in Mouse Adipocytes

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Abstract Fucoidan (fucan sulfate) is a fucose-containing sulfated polysaccharide from brown algae such as *Fucus vesiculosus*, *Ecklonia kurome*, and *Cladosiphon okamuranus*. The aim of this study was to investigate the effect of fucoidan on the expression of diabetes-related genes in mouse cell line 3T3-L1. 3T3-L1 adipocytes were cultured for 48 hr with or without fucoidan (10, 100, and 500 ppm) on a 60 mm dish. Reverse transcription polymerase chain reaction (RT-PCR) was used for measurement of peroxisome proliferators activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα), and glucose transporter 4 (GLUT4) RT-PCR analysis revealed that expression level of GLUT4, PPARγ, and C/EBPα mRNAs increased with fucoidan treatment from 10 to 500 ppm in a dose-dependent manner. Fucoidan appears to enhance insulin sensitivity by increasing the expression level of diabetes-related genes in 3T3-L1 adipocytes. Therefore, fucoidan is potentially useful as a natural therapeutic material for hyperglycemia in type II diabetes patients.

Keywords: fucoidan, 3T3-L1 cells, gene expression, PPARγ, C/EBPα, GLUT4

Introduction

Algae like *Undaria pinnatifida, Laminaria, Hizikia fusiforme*, and *Gelidium amansii* are popular foods in East Asia. These edible algae have been used as rich sources of minerals, vitamins, and dietary fiber. They have attracted attention recently as multifunctional foods for maintaining human health.

Fucoidan is a fucose-containing sulfated polysaccharide from brown algae species such as *Fucus vesiculosus*, *Ecklonia kurome*, and *Cladosiphon okamuranus* (1-3). For many years, fucoidans were regarded only as potential sources of L-fucose. In recent years fucoidan has been extensively studied due to its numerous biological activities including anticoagulant, antithrombotic, antitumor, antiviral, anticomplement, and antiinflammatory activities (3-8). But the antidiabetic effect of fucoidan has not been yet reported.

Diabetes mellitus is a multi-factorial and complex metabolic disease, characterized by elevated blood glucose concentrations. Type II diabetes mellitus is a disorder characterized by increased fasting blood glucose concentration, and is caused by suboptimal insulin production as well as impaired insulin function. The increasing prevalence of type II diabetes has reached crisis levels, as approximately 4.5 million Korean people suffer from this condition (9).

At present, pharmacological therapy of type II diabetes mellitus has been developed on several approaches intended to reduce the hyperglycemia that increases insulin release from pancreatic islets. Metformin, a reducing agent of hepatic glucose production and peroxisome proliferators activated receptor γ (PPAR γ) agonists, enhances insulin activity. However, most pharmacological therapies have several side effects such as hyperglycemia, edema, weight gain, and anemia (10, 11). On the other hand, the blood glucose-lowering effect of polysaccharides from mushrooms and seaweed has been investigated frequently (12, 13). However, there is only limited data on the antidiabetic biological mechanism of polysaccharides.

The CCAAT/enhancer binding proteins α (C/EBPα) are a family of basic leucine zipper (b-ZIP) transcription factors (14). The different C/EBP isoforms readily form heterodimers with each other and exhibit very similar DNA-binding activities. They are also all capable of transactivating a number of genes whose expression is increased during adipocyte differentiation (15). C/EBPα is one of the most important isoforms because it is a transcriptional factor suggested to play a key role in adipocyte differentiation. Expression of C/EBPα increases during the terminal differentiation of preadipocytes to adipocytes. C/EBPα possesses many characteristics required as a master regulator in cell culture models and coordinately activates transcription of many adipocyte genes (16). Expression level of C/EBPα, which might be involved in adipogenesis, is high in adipose tissue and the induction of its expression has been observed during differentiation of 3T3-L1 preadipocytes (17-19).

The peroxisome proliferator-activated receptor (PPARγ) is a subfamily of the nuclear hormone receptor superfamily. They have been shown to play important roles in regulating adipogenesis, energy balance, lipid metabolism, and glucose homeostasis (20, 21). PPARγ has been shown to be expressed in an adipose-specific manner and functions as a key regulator in adipocyte differentiation (22-27).

The ability of insulin to lower blood glucose level is due

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Received August 1, 2006; accepted September 7, 2006

in part to its inhibition of hepatic glucose output and its actions in muscle and fat to enhance glucose uptake through the GLUT4 (28). It has been shown that the selective loss of GLUT4 in adipose tissue or skeletal muscle leads to insulin resistance followed by diabetes mellitus in mice (29, 30). The latest reports have also provided evidence that insulin-induced glucose transport in fat tissue plays a key role in whole-body glucose homeostasis (29). On the other hand, high expression of GLUT4 in adipose tissue has been shown to enhance body insulin sensitivity in mice (31). At the molecular level, peripheral insulin resistance has been associated with defects in the transporter proteins transporting the glucose from intracellular pools to the plasma membrane, and with decreases in the numbers of insulin receptors (32). A glucose transporter protein of about five hundred amino acid residues is presented in most cells and plays an important role in passive facilitated diffusion.

Currently, fucoidan is used extensively as a foodstuff, food additive, and dietary supplement. The aim of this study is to provide basic data on antidiabetic activity that will predict the usefulness of fucoidan as a functional food material for the treatment of diabetes mellitus.

Materials and Methods

Materials 3T3-L1 was obtained from American Type Culture Collection (ATCC, CL-173) Dulbecco's modified Eagle's medium (DMEM), bovine serum (BS), fetal bovine serum (FBS), phosphate buffered saline (PBS), and trypsin-EDTA were purchased from Gibco (Gaithersburg, MD, USA). Penicillin-streptomycin was purchased from JBI (Daegu, Korea). Dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin, oil red O, and fucoidan (from *Fucus vesiculosus*) were purchased from Sigma (St. Louis, MO, USA). The chemical composition of fucoidan was as follows: total sulfur content was 8%, sodium content was 6%, and calcium content was 0.1%. Its molecular weight was determined to be 27,400 by multi-angle laser light scattering. Fucoidan was dissolved in distilled water for cell treatment. RNA*later* kit was from Ambion (Austin, TX, USA) and RNeasy mini kit was from Qiagen (Hilden, Germany). First-strand cDNA synthesis kit was purchased from Invitrogen (Carlsbad, CA, USA) and Taq DNA polymerase was obtained from Solgent (Daejeon, Korea). Primers were supplied by Bioneer (Daejeon, Korea).

Cell culture Murine preadipocytes were cultured, maintained, and differentiated as described by Student *et al.* (33, 34). Briefly, cells were plated and grown for 2 days post-confluence in DMEM with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 10% bovine calf serum (BCS/DMEM). Adipocyte differentiation was induced by treatment of confluent cells with a hormone mixture containing 10% FBS, 10 μg/mL insulin, 0.5 mM IBMX, and 0.25 μM Dex (MDI/FBS/DMEM)). Two days later, the growth medium was replaced with DMEM supplemented with only 10 μg/mL insulin and 10% FBS (I/FBS/DMEM). This medium was then replenished every other day. The concentrations of fucoidan treatment for 80% differentiation of adipocytes were 0, 10, 100, and 500 ppm. The same concentration of fucoidan was supplemented at 2 days intervals when culture medium was replenished.

RNA extraction Total RNA was extracted from 3T3-L1 adipocyte cells using a Qiagen RNeasy kit (Qiagen, Valencia, CA, USA). RNA quality was assessed by gel electrophoresis. RNA samples with an $O.D._{260/280}$ ratio higher than 2.0 were used for semi-quantitative reverse transcriptase polymerase reaction (RT-PCR).

Semi-quantitative RT-PCR RT-PCR was performed by QIAGEN OneStep RT-PCR kit using a PCR DNA Thermal Cycler (Corbett, San Francisco, CA, USA). First-strand cDNA was synthesized from total RNA at 50°C for 30 min using activated HotStraTaq DNA Polymerase by heating at 95°C for 15 min. PCRs of first-strand cDNA were performed by using 35 cycles (25 cycles β-actin) of 30 sec at 94°C, 30 sec at 50-68°C (depending on gene), and 1 min at 72°C cycle. This was followed by extension for 10 min at 72°C. All primers used in this study are listed in Table 1. RT-PCR products were subjected to electrophoresis on 2% agarose gels. β-Actin was used as a housekeeping gene for quantitative PCR to ensure that equal amounts of reverse-transcribed cDNA were applied to the PCR reaction. About 30 amplification cycles were performed by using the PCR protocol described above. The intensity of the PCR products on the agarose gel was measured with the Bio-1d program (Vilber Lourmat, GI-070AP).

Statistical analysis All measurements were repeated three times. The results were statistically analyzed by ANOVA and Duncan's multiple range tests. Statistical significance was accepted at a level of $p<0.05$ (SAS Inst., Inc., Cary, NC, USA).

Results and Discussion

Effect of fucoidan on adipocyte differentiation Confluent monolayers of 3T3-L1 cells (Fig. 1A) were treated with MDI/FBS/DMEM for 48 hr, transferred to I/FBS/DMEM medium, and cultured for an additional 48 hr. They began to accumulate small lipid droplets, at which time approximately 90% of the cells expressed the adipocyte phenotype (Fig. 1C). Adipocytes were treated for 48 hr after induction of differentiation with fucoidan at 0, 10, 100, or 500 ppm. The 3T3-L1 preadipocytes (Fig. 1B) differentiated into adipocytes. These 3T3-L1 cells changed the expression of diabetes mellitus-related genes as evaluated using specific primers for RT-PCR. We used β-actin, C/EBPα, PPARγ, and GLUT4 for RT-PCR (Table 1). The use of housekeeping gene β-actin was to demonstrate similar amounts of mRNA in each sample. We were thereby able to examine the effects of fucoidan on differentiation of adipocytes through the expression level of C/EBPα, PPARγ, and GLUT4 genes. These genes are important adipogenic transcription factors and are related to diabetes mellitus.

Effect of fucoidan on C/EBPα gene expression The effects of fucoidan on expression of C/EBPα genes were investigated by RT-PCR. The mRNA expression of C/

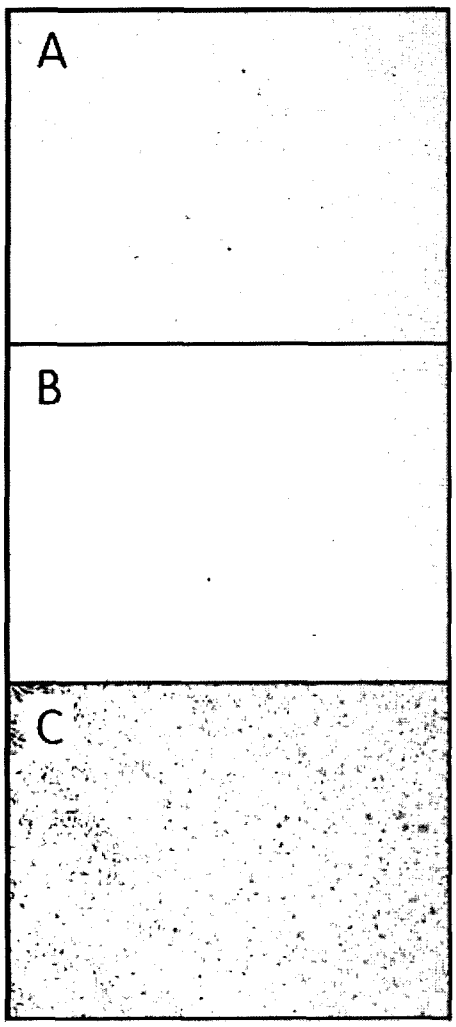

Fig. 1. Morphology of 3T3-L1. (A) Post-confluent, (B) Preadipocytes fibroblast shape, (C) Adipocytes lipid droplet, the nucleus and the cells become spherical in shape.

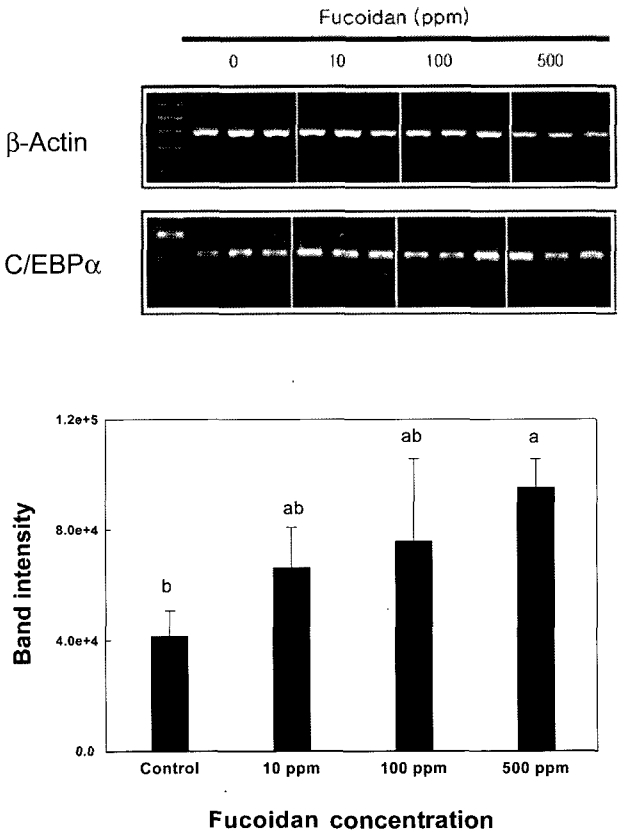

Fig. 2. Effect of fucoidan on C/EBPα gene expression. (Top) Gel electrophoresis of the expression of C/EBPα in 3T3-L1 adipocyte. (Bottom) Data is expressed as mean±SD values (n=3). Means are significantly different by Duncan's multiple range tests ($p<0.05$).

EBPα in 3T3-L1 was increased by fucoidan treatment at concentrations between 0 to 500 ppm.

Fucoidan was shown to enhance adipogenesis when cells were treated in stages of the terminal differentiation course (Fig. 2). The expression levels of C/EBPα mRNA were increased by a factor of 0.6 by fucoidan treatment at 10 ppm, 0.8 at 100 ppm, and 1.3 fold at 500 ppm ($p<$ 0.05). C/EBPα has multiple roles in adipocyte differentiation including coordinate transcriptional activation of a group of adipogenesis genes (16, 17). The first indication that C/EBPα might be involved in adipogenesis was its high level of expression in adipose tissue and the induction of its expression during differentiation of 3T3-L1 (17, 35). Induction of C/EBPα is reported to cause a transcriptional activation of a large group of adipocyte genes such as GLUT4, PPARγ, and SCD-1 (17, 36, 37).

Effect of fucoidan on PPARγ gene expression To determine the functional role of fucoidan in mature adipocytes, we studied 3T3-L1 adipocytes that had been

Table 1. Primer sequences for RT-PCR analysis

Primers	Sequence	Tm (°C)	Size (bp)
GLUT4			
Forward	**5'-TACTCATTCTTGGACGGTTC-3'**	52	288
Reverse	5'-TGATGTAGAGGTATCTGGGG-3'		
PPARγ			
Forward	**5'-TGAACGTGAAGCCCATCGAGGAC-3'**	65	119
Reverse	5'-TCTGTCATCTTCTGGAGCACCTTGG-3'		
C/EBPα			
Forward	**5'-AGGCTGTGCTGTCCCTGTATGC-3'**	63	395
Reverse	5'-ACCCAAGAAGGAAGGCTGGAAA-3'		

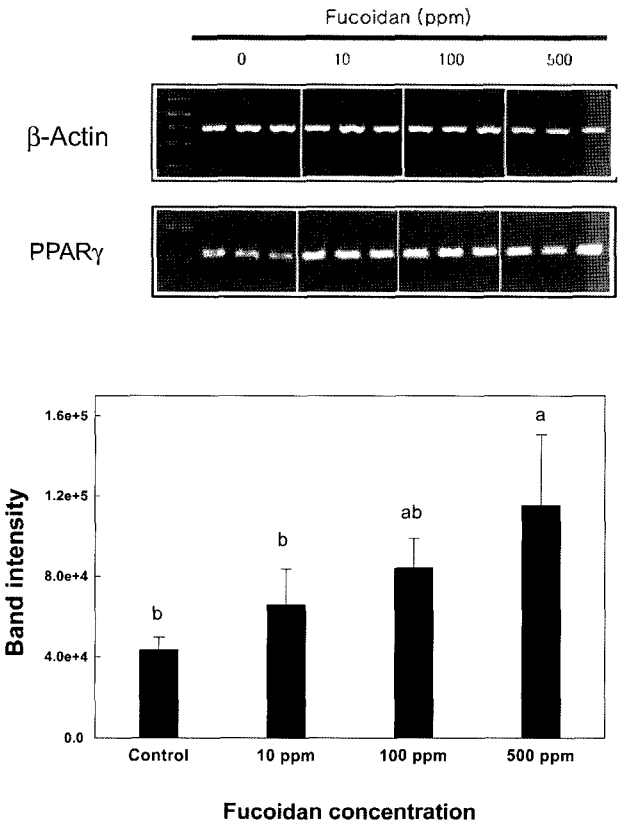

Fig. 3. Effect of fucoidan on PPARγ gene expression. (Top) Gel electrophoresis of the expression of C/EBPα in 3T3-L1 adipocyte. (Bottom) Data is expressed as mean±SD values (n=3). Means are significantly different by Duncan's multiple range tests ($p<0.05$).

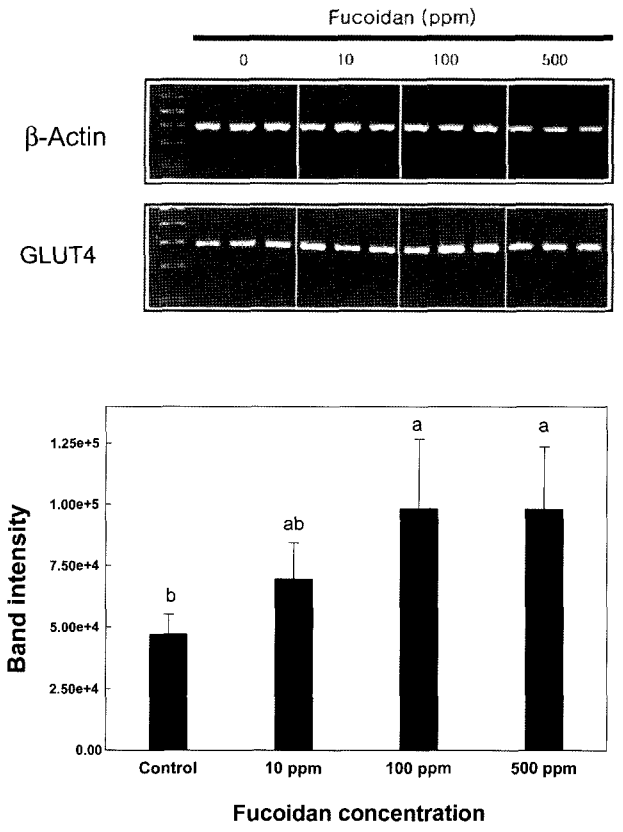

Fig. 4. Effect of fucoidan on GLUT4 gene expression. (Top) Gel electrophoresis of the expression of C/EBPα in 3T3-L1 adipocyte. (Bottom) Data is expressed as mean±SD values (n=3). Means are significantly different by Duncan's multiple range tests ($p<0.05$).

cultured for >13 days after the induction of differentiation. 3T3-L1 cells subjected to such long-term incubation can be considered mature adipocytes. The expression of mRNA to adipocyte differentiation was changed after 13 days culture, and then 3T3-L1 cells were treated with fucoidan for 48 hr. Over expression of PPARγ increased significantly both cell size and intracellular triglyceride content. Figure 3 shows that the expression level of PPARγ gene was changed by fucoidan treatment at 10, 100, and 500 ppm in 3T3-L1 adipocyte cells as evaluated by band intensity on RT-PCR. The band intensity of PPARγ gene increased in direct proportion to fucoidan concentration. The band intensities of the PPARγ gene at 100 and 500 ppm fucoidan were significantly higher than at control levels or at 10 ppm fucoidan ($p<0.05$). These results suggest that fucoidan enhances PPARγ gene expression.

The importance of PPARγ in adipocyte biology is underscored further by *in vivo* studies in which mutant mice lacking PPARγ displayed adipocyte hypocellulosity (38). The thiazolidinedione antidiabetics (TZDs) suppress insulin resistance in adipose tissue. They have the same effect in liver and skeletal muscle, which contain low concentrations of PPARγ (39). Consistent with these observations, Adipose specific PPARγ null mice have elevated levels of circulating lipids, increased hepatic gluconeogenesis, and insulin resistance (38). Additional evidence indicates that adipose tissue is the primary target for the systemic insulin-sensitizing actions of PPARγ ligands. Most notably, TZDs treatment improves the insulin resistance of partially lipoatrophic mouse (18). Furthermore, the activation of PPARγ is related to activation of key regulator gene that promotes a combination of lipid storage and lipogenesis genes such as CD36, aP2, ACS, and HSL. This activation causes whole body lipid repartitioning by increasing the triglyceride content of adipose tissue and lowering free fatty acid uptake. Triglyceride storage in mature adipocytes improves insulin sensitivity (40).

Effect of fucoidan on GLUT4 gene expression The effect of fucoidan on GLUT4 expression in 3T3-L1 adipocytes was examined. Differentiated adipocytes were treated for 48 hr after differentiation was induced with fucoidan at 0, 10, 100, or 500 ppm. The gene expression pattern of GLUT4 was evaluated by band intensity of electrophoresis. Expression levels of GLUT4 at 10, 100, and 500 ppm fucoidan treatment increased in a dose-dependent manner (Fig. 4). The band intensity of the GLUT4 gene increased with fucoidan concentration and was significantly higher at 100 and 500 ppm fucoidan than control or 10 ppm treatments ($p<0.05$). This data suggests that fucoidan can play a role as an insulin sensitizer through regulation of GLUT4 expression.

Both the reduction of GLUT4 in adipose tissue and the reduction of insulin-mediated recruitment of GLUT4 from intracellular vesicles to the plasma membrane are strongly associated with obesity and type II diabetes (41, 42). Accordingly, modulation of pathways involved in this unregulation may improve insulin sensitivity and reduce the hyperglycemia seen in type II diabetes. The target of insulin action is its ability to stimulate glucose uptake (43). Glucose uptake is mediated by glucose transporters such as GLUT1, GLUT2, GLUT3, and GLUT4. Glucose transporter 4 is one of the members of the glucose transporter family expressed on 3T3-L1 adipocytes.

In conclusion, all of this data suggests that fucoidan has a role as a naturally occurring PPARγ agonist that can contribute to the insulin-sensitizing effects of C/EBPα and PPARγ through regulating lipid and glucose metabolism in 3T3-L1. Therefore, fucoidan might enhance insulin sensitivity by increasing GLUT4 expression in adipocytes. Therefore, fucoidan might have potential as a natural medicinal material for treating hyperglycemia in type II diabetes patients.

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