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Fucoidan from sea cucumber *Cucumaria frondosa* exhibits anti-hyperglycemic effects in insulin resistant mice via activating the PI3K/PKB pathway and GLUT4

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The present study investigated the anti-hyperglycemic properties and mechanisms of fucoidan, isolated from *Cucumaria frondosa* (*Cf*-FUC), in insulin resistant mice. Male C57BL/6J mice were fed regular diet or high-fat/high-sucrose diet for 19 weeks. Model animals were dietary administrated either rosiglitazone (RSG, 1 mg/kg·bw), fucoidan (*Cf*-FUC, 80 mg/kg·bw) or their combinations. Results showed that *Cf*-FUC significantly reduced fasting blood glucose and insulin levels, and enhanced glucose tolerance and insulin tolerance in insulin-resistant mice. Quantitative real-time PCR analysis showed that *Cf*-FUC increased the mRNA expressions of insulin receptors (IR), insulin receptor substrate 1 (IRS-1), phosphatidylinositol 3 kinase (PI3K), protein kinase B (PKB), and glucose transporter 4 (GLUT4). Western blot assays demonstrated that *Cf*-FUC showed no effect on total protein expression but nevertheless enhanced the phosphorylation of proteins listed above and increased translocation of GLUT4 to the cell membrane. Furthermore, *Cf*-FUC enhanced the effects of RSG. These results indicated that *Cf*-FUC exhibited significant anti-hyperglycemic effects via activating PI3K/PKB pathway and GLUT4 in skeletal muscle and adipose tissue.

[**Key words:** Sea cucumber fucoidan; Insulin resistance; Anti-hyperglycemic effects; Phosphatidylinositol 3 kinase pathway; Glucose transporter 4]

Diabetes mellitus is a serious, non-contagious disorder characterized by mellithemia due to an absolute or relative deficiency of insulin secretion or insulin resistance (1). In particular, type 2 diabetes is increasingly associated with morbidity and mortality world-wide (2). However, current therapies are often associated with inadequate glycemic control, adverse events and high secondary-failure rates (3). For instance, rosiglitazone (RSG) is an oral antidiabetic agent that can maintain glucose homeostasis and improve insulin resistance, but it also suffers from generally inadequate efficacy and a range of serious adverse effects (4,5). Therefore, seeking for safe and effective dietary regulations focused on ameliorating type 2 diabetes has been a hot spot of research. Hyperglycemia is the main pathological effect of type 2 diabetes and insulin resistance is the major risk factor for developing type 2 diabetes (6,7). Thus, alleviating insulin resistance is an important preventative step in halting the development of hyperglycemia and ultimately type 2 diabetes. In insulin resistance, target tissues respond poorly to the normal levels of the circulating hormone, which therefore fails to regulate the glucose homeostasis in skeletal muscle, liver, and adipose tissues (8). A fundamental mechanism for antagonizing insulin resistance is the rapid action of insulin to stimulate glucose uptake and metabolism in peripheral tissues which relied on the regular insulin signaling (9). Rains and Jain (10) confirmed that the insulin signal is amplified through the PI3K/PKB pathway. Skeletal muscle is considered the most important tissue in terms of glucose storage, because it accounts for approximately 75% of the total insulin-dependent glucose uptake in both human and rodents (11). Adipose tissue is another important destination and insulin insensitivity here plays a central role in the development of the overall insulin resistance, type 2 diabetes and cardiovascular diseases (12).

Sea cucumber has been historically used in China because of its numerous health benefits. It contains various bioactive components such as collagen polypeptide, polysaccharide, saponins, and lipids (13–16). Fucoidan isolated from the sea cucumber (SC-FUC) is a sulfated polysaccharide containing a substantial percentage of L-fucose and sulfate ester groups (17). SC-FUC has been reported to have anticoagulant and antithrombotic properties (18), protection from gastric damage (19) and inhibition for osteoclastogenesis (20). In addition, multiple reports showed that fucoidan isolated from alga exhibited anti-hyperglycemia effects, for example, fucoidan extracted from *Saccharina japonica* had a pronounced hypoglycemic effect in alloxan-induced diabetic rats (21) and fucoidan derived from the *Sporophyll* of *Undaria pinnatifida* could regulate blood glucose homoeostasis in C57BL/KSJm+/+db and C57BL/KSJ db/db mice (22), while the literature on SC-FUC is scarce. We therefore isolated SC-FUC from *Cucumaria frondosa* (*Cf*-FUC) and demonstrated its anti-hyperglycemic potential in combination with RSG, which is a representative of classic hypoglycemic drugs. To investigate the mechanism of action, we determined the insulin-mediated genes mRNA expression and protein phosphorylation levels of the classic PI3K/PKB pathway and translocation of GLUT4 protein in insulin resistant mice.

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MATERIALS AND METHODS

Preparation of the *Cf*-FUC Dry sea cucumber *C. frondosa* was purchased from a marketplace (Qingdao, Shandong Province, China) and authenticated by Prof. Yulin Liao from Institute of Oceanology, Chinese Academy of Science. The preparation of Cf-FUC was performed according to the method described by Chang et al. (17). Briefly, the dry body wall of the sea cucumber was grinded and degreased with acetone. After hydrolysis with papain and precipitation with cetylpyridinium chloride, the crude polysaccharide was acquired and eluted in Sepharose Q Fast Flow column (GE Healthcare, Uppsala, Sweden) with linear gradient of NaCl solution. The eluent containing fucoidan was detected by chromatography (Aglient1100, Agilent Technologies, Santa Clara, CA, USA), then collected, dialyzed (10 kDa cutoff) and lyophilized. Pre-column derivatization high performance liquid chromatography and ion chromatography revealed the sulfate content of the *Cf*-FUC was 29.31% and the monosaccharide compositions were fucose, galactosamine, galactose and glucosamine, with a ratio of 1: 0.1: 0.3: 0.17.

Animal experiment Male C57BL/6J mice, 4–5 weeks, were purchased from Vital River Laboratory Animal Center (Beijing, China; licensed ID: SCXK2007-0001) with free access to distilled water in a 12–12 h light–dark condition at 23 ± 1°C. All animals experiments were conducted in accordance with internationally valid guidelines and experimental protocols with prior approval by the animal ethics committee according to the guidelines of the Standards for Laboratory Animals of China (GB 14922-94, GB 14923-94, and GB/T 14 925-94).

The mice were randomly assigned to 6 groups of 10 animals each: normal control, model control, rosiglitazone group (RSG), *Cf*-FUC group, low-dose and high-dose combination groups (RSG+20*Cf*-FUC, RSG+80*Cf*-FUC). Administration groups above were dietary supplied with rosiglitazone (1 mg/kg·bw), *Cf*-FUC (80 mg/kg·bw) and combinations of low-dose or high-dose *Cf*-FUC (20 and 80 mg/kg·bw respectively) and rosiglitazone (1 mg/kg·bw). Mice in the normal control group were fed with a regular diet and the others were fed with high fat/sucrose diet (HFSD, according to the AIN-93 recipe). The dietary dose of *Cf*-FUC and rosiglitazone (Taji Group, Chongqing, China) as well as the forage compositions are shown in Table 1. During the experiment, the dietary dose of *Cf*-FUC and rosiglitazone was modified slightly according to the body weight and food intake to make a standardized comparison. The animals in each group were treated for 19 weeks. Body weight and food intake were measured during the experiment. Glucose tolerance and insulin tolerance tests were performed 10 days and 5 days before the last treatment respectively. After the last treatment, the hind limb skeletal muscle and epididymal adipose tissue were excised to analyze the PI3K/PKB pathway gene-expression levels, as well as GLUT4 translocation.

Glucose tolerance tests Blood was collected from the tail veins of mice after an 8-h fasting period. Fasting blood glucose levels were determined using a commercial testing kit (Biosino Bio-technology and Science Inc., Beijing, China). Oral glucose tolerance test was conducted by measuring the blood glucose levels at 0, 0.5, 1 and 2 h after gavage of glucose at a dose of 2 g/kg·bw. The value was negatively estimated using area under curve (AUC), which was calculated as listed below.

$$\mathrm{AUC} = 0.25 \times \mathrm{A} + 0.5 \times \mathrm{B} + 0.75 \times \mathrm{C} + 0.5 \times \mathrm{D} \quad (1)$$

where A, B, C and D represent glucose level at 0, 0.5, 1 and 2 h, respectively.

Insulin tolerance tests After an 8-h fasting period, blood was collected through the tail vein and separated to determine fasting blood glucose levels. Insulin (0.75 U/kg·bw, Sigma-Aldrich, St. Louis, MO, USA) was administered by intraperitoneal injection. Blood glucose was determined at 0, 30, 60, and 120 min. Changes in glucose were plotted over time; AUC was also calculated as described before.

Determination of insulin sensitivity Serum insulin level was assessed by insulin ELISA kit (R&D, Minneapolis, MN, USA). Homeostasis model assessment of insulin resistance index (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) (23) were calculated as follows:

$$\begin{aligned}\text{HOMA-IR} = {} & \text{fasting blood glucose level } (\text{mmol}\cdot\text{L}^{-1}) \\ & \times \text{serum insulin level } (\text{mIU}\cdot\text{ml}^{-1})/22.5\end{aligned} \quad (2)$$

$$\begin{aligned}\text{QUICKI} = {} & 1/(\lg(\text{fasting blood glucose level } (\text{mmol}\cdot\text{L}^{-1})) \\ & + \lg(\text{serum insulin level } (\text{mIU}\cdot\text{ml}^{-1})))\end{aligned} \quad (3)$$

Insulin stimulation and tissue processing The insulin stimulation was prepared according to the method of Wong et al. (24). At the end of 19 weeks, the animals were fasted overnight and injected intraperitoneally with 40 U of insulin (Sigma-Aldrich) per kg (n = 4 per group) for phosphorylated protein analysis or equivalent volumes of saline (n = 3 per group) for total protein levels and mRNA expression assays. After 5 min, the hind limb skeletal muscle and epididymal adipose tissue were excised and immediately frozen in liquid nitrogen and stored at −80°C.

Skeletal muscle and adipose tissue were prepared for GLUT4 translocation assay in a similar manner as for protein phosphorylation, except that the fasted animals were injected with 0.5 U/kg insulin and sacrificed 30 min after injection. Skeletal muscle plasma membrane and adipose tissue plasma membrane were prepared according to the method as described previously (25,26). Briefly, 3 g of frozen skeletal muscle tissue was homogenized at low-speed in ice-cold lysis buffer. The homogenate was triple-centrifuged at 1200×*g*, 9000×*g*, and 190,000×*g* successively to obtain pellets containing plasma membranes. The pellets were further separated by sucrose-gradient (25%, 32%, and 35% wt/wt) centrifugation at 150,000×*g* for 16 h. Fractions containing membrane GLUT4 were collected from the fraction of 25% sucrose solution and subjected to 190,000×*g* for 1 h and used for Western blot analysis. Adipose tissue (3 g) was homogenized in TES buffer at 4°C. The homogenate was centrifuged at 3000×*g* to obtain the liquor and then centrifuged at 12,000×*g* to obtain the pellets containing the adipose tissue plasma membranes.

Quantitative real-time PCR analysis Total RNA was isolated from skeletal muscle and adipose tissue of mice using TRIzol reagent and the concentrations were detected by spectrophotometer. One microgram of RNA was converted to cDNA using M-MLV reverse transcriptase. Real-time PCR was conducted using the Bio-Rad iQ5 system (Bio-Rad, Hercules, CA, USA). Approximately 25 μl reaction volume was used for the quantitative real-time PCR assay which consisted of 12.5 μl Maxima SYBR Green qPCR Master mix, 10 μM primers (0.3 μl each of forward and reverse primer), 5.9 μl nuclease-free water and 6 μl template. The thermal procedure included an initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s and extension at 72°C for 30 s. Data normalization was conducted using β-actin as the endogenous reference. The gene expression levels were analyzed by relative quantification using the standard curve method. The sequences of the primers (Sangon Biotech, Shanghai, China) are described in Table 2.

Western blot analysis Approximately 50 μg of solubilized skeletal muscle protein was fractionated by electrophoresis on 1% SDS-PAGE gels. The fractionated

TABLE 2. Sequence of the primers used in the quantitative real-time PCR.

Gene	Forward primer	Reverse primer
IR	5′-CCTACTGCTATGGGCTTCG-3′	3′-GTTCTGGTCTGGGCTTCTA-5′
IRS-1	5′-TTGCTTGGCACAATGTAGAA-3′	3′-GAGGATCGTCAATAGCGTAAC-5′
PI3K	5’-CCCATACAAGGTGTTAGCC-3′	3′-ACTCTGACCTGGGATACCG-5′
PKB	5′-CCAGATGGTAGCCAACAGT-3′	3′-GATAGAGTTTGAGGAGCCG-5′
GLUT4	5′-ACTAAGAGCACCGAGACCAA-3′	3′-CTGCCCGAAAGAGTCTAAAG-5′
β-Actin	5′-CAAGGCATTGCTGACAGGATG-3′	3′-GGTCGTCTACACCTAGTCGT-5′

TABLE 1. Ingredients of the mice forage (g/kg diet).[a,b]

Composition	Normal control	Model control	RSG	80*Cf*-FUC	RSG + 20*Cf*- FUC	RSG + 80*Cf*- FUC
Casein	200	200	200	200	200	200
Cornstarch	650	250	249.925	248.8	249.625	248.725
Sucrose	0	200	200	200	200	200
Corn oil	50	50	50	50	50	50
Lard	0	200	200	200	200	200
Mineral mix	35	35	35	35	35	35
Vitamin mix	10	10	10	10	10	10
Cellulose	50	50	50	50	50	50
Choline Bitartrate	3	3	3	3	3	3
DL-Methionine	2	2	2	2	2	2
Rosiglitazone	–	–	0.075	–	0.075	0.075
Cf-FUC	–	–	–	1.2	0.3	1.2

[a] RSG was fed in dose of 1 mg/kg·bw everyday.
[b] Mineral mix and vitamin mix were prepared according to AIN-93 recipe.