



Fish oil prevents excessive accumulation of subcutaneous fat caused by an adverse effect of pioglitazone treatment and positively changes adipocytes in KK mice

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ABSTRACT

Pioglitazone, a thiazolidinedione (TZD), is widely used as an insulin sensitizer in the treatment of type 2 diabetes. However, body weight gain is frequently observed in TZD-treated patients. Fish oil improves lipid metabolism dysfunction and obesity. In this study, we demonstrated suppression of body weight gain in response to pioglitazone administration by combination therapy of pioglitazone and fish oil in type 2 diabetic KK mice. Male KK mice were fed experimental diets for 8 weeks. In safflower oil (SO), safflower oil/low-dose pioglitazone (S/PL), and safflower oil/high-dose pioglitazone (S/PH) diets, 20% of calories were provided by safflower oil containing 0%, 0.006%, or 0.012% (wt/wt) pioglitazone, respectively. In fish oil (FO), fish oil/low-dose pioglitazone (F/PL), and fish oil/high-dose pioglitazone (F/PH) diets, 20% of calories were provided by a mixture of fish oil and safflower oil. Increased body weight and subcutaneous fat mass were observed in the S/PL and S/PH groups; however, diets containing fish oil were found to ameliorate these changes. Hepatic mRNA levels of lipogenic enzymes were significantly decreased in fish oil-fed groups. These findings demonstrate that the combination of pioglitazone and fish oil decreases subcutaneous fat accumulation, ameliorating pioglitazone-induced body weight gain, through fish oil-mediated inhibition of hepatic *de novo* lipogenesis.

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Abbreviations: AOX, acyl-CoA oxidase; ACC, acetyl-CoA carboxylase; ATM, adipose tissue macrophage; AUC, area under the curve; BAT, brown adipose tissue; CPT-1, carnitine palmitoyl transferase 1; CT, computed tomography; DHA, docosahexaenoic acid; ELISA, enzyme-linked immunosorbent assay; EPA, eicosapentaenoic acid; FAS, fatty acid synthase; FFA, free fatty acid; G6pase, glucose-6-phosphatase; GPAT, glycerol-3-phosphate acyltransferase; HDL-C, high-density lipoprotein cholesterol; H&E, hematoxylin and eosin; HOMA-IR, homeostasis model assessment of insulin resistance; Insig-1, insulin-induced gene 1; IR, insulin resistance; ITT, insulin tolerance test; MCAD, medium-chain acyl-CoA dehydrogenase; MCP-1, monocyte chemoattractant protein-1; OGTT, oral glucose tolerance test; PEPCK, phosphoenolpyruvate carboxykinase; PPAR α , peroxisome proliferator-activated receptor alpha; PPAR γ , peroxisome proliferator-activated receptor gamma; RT-PCR, real-time polymerase chain reaction; SCD-1, stearoyl-CoA desaturase 1; SREBP, sterol regulatory element-binding protein; TNF- α , tumor necrosis factor- α ; TLR-4, toll-like receptor-4; TZD, thiazolidinedione; UCP-2, uncoupling protein 2; VLDL, very low-density lipoprotein; WAT, white adipose tissue.

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1. Introduction

Lifestyle-related diseases, particularly type 2 diabetes, are increasingly becoming a major global health issue associated with excessive caloric intake and decreasing physical activity [1]. Type 2 diabetes epidemic has grown rapidly worldwide and is known to affect an estimated 387 million people (Diabetes Atlas 2014 update, International Diabetes Federation, IDF, 2014). The major pathogenic mechanism underlying type 2 diabetes is thought to be insulin resistance (IR) associated with abdominal obesity [2]. Excessive amounts of unconsumed calories result in triglyceride accumulation in adipocytes, particularly in visceral fat tissue, leading to cellular hypertrophy and stimulation of inflammatory cytokine production [3–5]. In addition, large amounts of free fatty acids (FFAs) derived from hypertrophic adipocytes are transferred to insulin responsive organs, such as the liver, skeletal muscle, and pancreas, as “ectopic fat” leading to decreased insulin sensitivity [6,7]. Adiponectin, an adipose tissue-derived secreted protein with insulin-sensitizing activity, has been shown to be decreased in abdominal obesity, with a negative correlation found between

Table 1
Composition of experimental diets.

Group	Ingredients (g)					
	SO	S/PL	S/PH	FO	F/PL	F/PH
Safflower oil	8	8	8	4	4	4
Fish oil				4	4	4
Casein	20	20	20	20	20	20
Sucrose	10.37	10.37	10.37	10.37	10.37	10.37
β -starch	51.83	51.83	51.83	51.83	51.83	51.83
Vitamin mix ^a	1	1	1	1	1	1
Mineral mix ^a	3.5	3.5	3.5	3.5	3.5	3.5
Cellulose powder	5	5	5	5	5	5
L-Cystin	0.3	0.3	0.3	0.3	0.3	0.3
<i>t</i> -Butylhydroquinone	0.0016	0.0016	0.0016	0.0016	0.0016	0.0016
Pioglitazone		0.006	0.012		0.006	0.012
Total	100.00	100.01	100.01	100.00	100.01	100.01
Energy (kcal/100 g)	374.02	374.00	373.99	374.02	374.00	373.99
Fat energy (%)	19.70	19.70	19.70	19.70	19.70	19.70

^a Vitamin and mineral mix were based on the AIN-93 M formation. Vitamin mix substituted 0.25% sucrose for choline bitartrate.

plasma adiponectin levels and body mass index [8]. Through the interactions of these complex mechanisms, obesity-induced accumulation of visceral fat is thought to contribute to IR through “lipotoxicity” [2].

Thiazolidinediones (TZDs), potent and selective ligands for peroxisome proliferator-activated receptor gamma (PPAR γ), are widely used clinically in the treatment of type 2 diabetes as insulin-sensitizing agents [9]. TZDs promote the differentiation of preadipocytes into mature adipocytes and apoptosis of hypertrophic adipocyte, a process termed “adipose tissue remodeling.” Further, TZDs increase plasma adiponectin levels and reduce the production of adipocytokines, such as tumor necrosis factor- α (TNF- α) and monocyte chemoattractant protein-1 (MCP-1), thereby ameliorating adipocytokine-mediated IR [10–13]. Thus, the antihyperglycemic effects of TZDs are thought to be mediated independently of increased insulin production, suggesting the greatest utility of TZDs lies in the treatment of type 2 diabetic patients without pancreatic beta cell failure [14,15]. Many studies have demonstrated TZDs have multifaceted utility including the improvement of lipid profiles [16,17], amelioration of nonalcoholic steatohepatitis [18,19], systemic anti-inflammatory effects [20], and the prevention of arteriosclerosis [21,22]. And, TZD-induced “adipose tissue remodeling” is thought to lead increased absorption and storage of excess lipids by small adipocytes newly derived in response to the effects of TZDs [13,23]. However, body weight gain is considered a major adverse effect of TZD therapy following results from animal studies and several clinical trials in patients with type 2 diabetes [24,25]. Moreover, subcutaneous fat accumulation is frequently observed in patients administered TZDs [26].

Fish oil contains eicosapentaenoic acid (EPA, 20-5) and docosahexaenoic acid (DHA, 22-6), which have been shown to have beneficial effects in hyperlipidemia, fatty liver, atherosclerosis, and cardiovascular disease in both animal models and clinical trials [27–30]. Hepatic *de novo* lipogenesis is mainly controlled by sterol regulatory element-binding proteins (SREBPs), transcription factors that regulate the expression of genes involved in lipogenesis. SREBP-1c, one of the SREBPs isoforms, plays a particularly critical role in fatty acid synthesis through the regulation of several target genes including fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase 1 (SCD-1) [31]. Fish oil consumption has been shown to decrease mRNA expression and mature protein levels of SREBP-1c [32,33]. Additionally, fatty acid oxidation has been shown to be stimulated by fish oil consumption through stimulation of peroxisome proliferator-activated receptor alpha (PPAR α), a nuclear receptor involved in the regulation of numerous target genes, such as acyl-CoA oxidase (AOX) and uncou-

pling protein 2 (UCP-2) [32,34]. In our previous study of female KK mice, we demonstrated a diet with 25% of total calories from fish oil had numerous beneficial effects including suppression of body weight gain associated with whole-body adiposity, improvements in lipid metabolism dysfunction, the prevention of hyperinsulinemia due to increased fatty acid oxidation, and decreased lipid synthesis [35]. Therefore, we hypothesized that the combination of pioglitazone and fish oil prevents pioglitazone-induced body weight gain attributed to the accumulation of subcutaneous fat and exerts synergistic beneficial effects on glucose and lipid metabolism. In this study, we evaluated the combined effects of pioglitazone and fish oil in mice of type 2 diabetes to test the above hypothesis and inform the development of novel TZD treatment approaches.

2. Materials and methods

2.1. Animals and diets

All animal studies were approved by the Guidelines of Institutional Animal Care and Use Committee at the Josai University Life Science Center performed in accordance with the “Standards Relating to the Care and Management of Experimental Animals” (Notice No. 6 of the Office of the Prime Minister of Japan dated March 27, 1980). Male KK mice at 6 weeks of age were obtained from Tokyo Laboratory Animals Science Co. (Tokyo, Japan). All mice were individually housed and allowed free access to water and feed under the conditions of a 12-h light–dark cycle, a temperature of $22 \pm 2^\circ\text{C}$, and humidity of $55 \pm 10\%$ at Josai University Life Science Center. Mice were fed a standard commercial rodent diet for 1 week to stabilize metabolic conditions and divided into 6 groups ($n = 5$ in each group). All groups were fed experimental diets with contents composed of 60% carbohydrates, 20% fats, and 20% protein for 8 weeks. Dietary fats used safflower oil (Benibana Foods, Tokyo, Japan) and/or fish oil (NOF, Tokyo, Japan). Safflower oil contained 78 wt% oleic acid; fish oil contained 40.4 wt% polyunsaturated fatty acids, especially 6.6 wt% EPA and 24.7 wt% DHA. SO diet contained 20% of calories from safflower oil, and FO diet contained 10% of calories from safflower oil and 10% from fish oil. SO and FO diets were supplemented with 0.006% wt/wt (low-dose pioglitazone [PL]) or 0.012% wt/wt (high-dose pioglitazone [PH]) pioglitazone and designated S/PL, S/PH, F/PL, or F/PH accordingly. Dietary details are shown in Table 1. The feed were changed once in every 2–3 days, and the residual amounts were recorded and showed as total food intake during experimental periods. All diets were stored at -30°C until each meal is supplied freshly.

2.2. Computed tomography

Radiographic estimations of abdominal composition were performed by computed tomography (CT) for experimental animals using the mouse mode of the CT scanner (La Theta LCT100; ALOKA, Tokyo, Japan). At the end of the experiment, mice fasted for 3 h were anesthetized with intraperitoneal injections of pentobarbital sodium (Dainippon Sumitomo Pharma, Osaka, Japan) before scanning. Abdominal compositions of visceral and subcutaneous fats were estimated by fat slice images at 2 mm intervals between the second lumbar vertebra (L2) and L4 using La Theta software (version 2.10).

2.3. Collection of blood and tissue samples

After CT scanning, blood samples were obtained from tail veins of anesthetized mice. Glucose levels were measured using a blood glucose monitoring system (One Touch Ultra; Johnson & Johnson, Inc.). Mice were then immediately weighed prior to dissection.

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