Impact of discontinuation of fish oil after pioglitazone-fish oil combination therapy in diabetic KK mice

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Abstract

Pioglitazone is one of the thiazolidinediones (TZDs) and an insulin-sensitive drug for type 2 diabetes. In our previous study, a combination of pioglitazone and fish oil rich in n-3 polyunsaturated fatty acids (PUFAs) was shown to inhibit pioglitazone-induced side effects, such as accumulation of subcutaneous fat and body weight gain. However, the effects of the discontinuation of fish oil after combination treatment with TZD and fish oil are not clear. In this study, discontinuation of fish oil for 4 weeks showed several unfavorable effects: 1) return of plasma adiponectin level; 2) reversal of the inhibition of lipogenesis and activation of fatty acid β-oxidation in liver; 3) increase in hypertrophic adipocytes in epidydimal white adipose tissue (WAT); and 4) accumulation of lipids in brown adipose tissue (BAT). However, insulin resistance was ameliorated by pioglitazone with or without fish oil treatment and the discontinuation of fish oil. These findings indicate that discontinuation of n-3 PUFA after combination therapy with TZDs adversely affects lipid metabolism and energy homeostasis in liver, epididymal WAT, and BAT.

1. Introduction

Thiazolidinediones (TZDs) are ligands of peroxisome proliferator-activated receptor-γ (PPARγ). These are insulin-sensitive drugs that have been clinically used for the treatment of type 2 diabetes. PPARγ is critical for adipocyte differentiation and maintenance of mature adipocytes. In addition, PPARγ regulates a variety of target genes involved in lipid and glucose metabolism, such as lipoprotein lipase, fatty acid transporter CD36, phosphoenolpyruvate carboxykinase (PEPCK), and adiponectin [1]. Previous studies have shown that TZDs induce the differentiation of preadipocytes into mature adipocytes and apoptosis of hypertrophic adipocytes [2, 3]. The former effect contributes to increased levels of plasma adiponectin, an adipokine that improves insulin sensitivity in skeletal muscle and suppresses hepatic gluconeogenesis [4-6]. The latter decreases the production of proinflammatory adipokines, such as tumor necrosis factor-α and monocyte chemoattractant protein-1, which lowers fasting glucose level by a mechanism independent of insulin secretion [7]. Thus, TZDs are associated with a low risk of hypoglycemia and pancreatic exhaustion. TZDs were also shown to protect against pancreatic β-cell dysfunction by reducing the generation of reactive oxygen species and ameliorating endoplasmic reticulum (ER) stress associated with over production of insulin [8, 9]. However, TZD therapy is associated with several adverse

effects such as heart failure, fluid retention, increase in body weight, bone fracture, and bladder cancer [10-14]. Furthermore, higher doses of TZDs are associated with a greater risk of adverse events [14-16]. Therefore, the identification of strategies to reduce dosage of TZDs is a key imperative.

In an attempt to enhance the safety and benefits of TZDs treatment, Majima et al. demonstrated that low-dose TZDs (7.5 mg/day) moderated body weight gain in patients with type 2 diabetes compared to the standard-dose (15 mg/day), while still showing a clinically significant hypoglycemic effect [17]. However, in another clinical study, 7.5 mg/day TZD moderated body weight gain, but failed to improve glucose tolerance [15]. Several *in vivo* animal studies and clinical studies have suggested that the combination of low-dose TZDs plus another antidiabetic drug, such as metformin or a dipeptidyl peptidase IV inhibitor, can achieve similar antidiabetic efficacy while preventing adverse events [18, 19]. In our previous study, a combination of TZD and fish oil rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was shown to prevent TZD-induced body weight gain by suppressing the accumulation of subcutaneous fat; in addition, it showed a protective effect against pancreatic β-cell dysfunction by reducing ER stress in diabetic KK mice [9, 20]. The KK mouse is an animal model of type 2 diabetes and considered to have the polymorphisms on leptin receptor gene, which

results in a part of cause for obesity and diabetic conditions [21]. The mice present many striking diabetic changes in pancreas, such as the hypertrophy and hyperplasia of the islets, the hypertrophy and degranulation of ß-cells [22]. In another study, we found that the antidiabetic efficacy of a combination of low-dose TZD and fish oil was similar to that of high-dose TZD without concomitant body weight gain in aged diabetic KK mice [23].

Although pharmacological treatment is the cornerstone of treatment of many diseases, the discontinuation of medication is recommended after the occurrence of adverse effects or negative effects [24, 25]. A systematic review also found that a discontinuation regimen is feasible in clinical settings [26]. On the other hand, several studies have shown that drug discontinuation aggravates the disorder and increases the risk of mortality [27, 28]. However, the effect of discontinuation of combination therapy with TZDs and n-3 polyunsaturated fatty acids (PUFAs) (such as EPA and DHA) is not well characterized.

The present study aimed to examine the influence of discontinuation of n-3 PUFAs after the treatment of type 2 diabetes with a combination of TZD and n-3 PUFAs. For this purpose, we evaluated the combination of pioglitazone (a TZD drug) and fish oil as a source of EPA and DHA in male KK mice of type 2 diabetes.

2. Methods

2.1 Animal and diets

This study was performed in accordance with the “Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions” (Ministry of Education, Culture, Sports, Science and Technology, Japan, Notice No. 71, dated June 1, 2006) and approved by the Institutional Animal Care and Use Committee of Josai University. Six-week-old male KK/Ta mice were purchased from Tokyo Laboratory Animals Science Co. (Tokyo, Japan) and fed a standard commercial diet (CE-2, Crea Japan, Inc.) for 1 week to acclimatize them to their new environment. All mice were maintained in a controlled environment [temperature, 22°C ± 2°C; humidity, 55% ± 10%; 12:12-h light-dark cycle (lights on: 7:00 AM–7:00 PM)] at Josai University Life Science Center and allowed ad libitum access to food and water. At 7 weeks of age, the mice (the body weight: 23.3～27.6g) were divided into four weight-matched groups (5 animals per group). The experimental diets were designed to achieve total fat energy level at 20 energy% (en%) (Supplementary Table 1). The control (Con) group was administered a control diet, which included 20 en% safflower oil (Benibana Foods Co. Ltd., Tokyo, Japan). The pioglitazone (P) group and the pioglitazone plus fish oil (PF) group received a control diet or a fish oil diet including

10 en% safflower oil and 10 en% fish oil (NOF Co., Tokyo, Japan), supplemented with 0.012 weight% (wt%) pioglitazone hydrochloride (Wako Pure Chemical Industries Ltd., Osaka, Japan), respectively. Mice in these three groups were fed their respective diets throughout the experimental period. The PF/P group was administered the pioglitazone plus fish oil diet (PF) during the first 8 weeks, and the pioglitazone plus control diet (P) during the last 4 weeks. The detailed experimental design is shown in Supplementary Fig. 1.

2.2 Computed tomography

At the end of the experiment, fasting mice were intraperitoneally injected with pentobarbital sodium (Kyoritsu Seiyaku Co., Tokyo, Japan) for anesthesia and underwent computed tomography (CT) scans at 2-mm intervals between the second and fourth lumbar vertebrae using a La Theta LCT100 scanner (Hitachi Aloka Medical Ltd., Tokyo, Japan). Abdominal visceral and subcutaneous fat content was estimated using the La Theta software (version 2.10). The scanned images were represented by pink (visceral fat) and yellow regions (subcutaneous fat), respectively.

2.3 Sample collection

After CT scanning, blood samples were obtained from the tail vein and glucose concentration and measured using a blood glucose monitor (One Touch Ultra; Johnson & Johnson, New Brunswick, NJ). Subsequently, the mice were weighed and blood samples removed from the inferior vena cava for biochemical assays. The liver, epididymal white adipose tissue (WAT), and interscapular brown adipose tissue (BAT) were removed. Tissue samples were immediately frozen using liquid nitrogen and stored at −80°C until further processing. All the samples were obtained from the fasting state.

2.4 Morphological analysis

For histopathological and morphometric analyses, portions of WAT were fixed in 10% neutral buffered formalin (Wako Pure Chemical Industries), embedded in paraffin, and stained with hematoxylin and eosin by Kotobiken Medical Laboratories Inc. (Tokyo, Japan). The mean adipocyte size in 7–11 randomly chosen fields of epididymal WAT specimens was evaluated for each group. The adipocyte areas were measured from more than 1000 cells per group using Image J software (Wayne Rasband, NIH).

2.5 Biochemical parameters

The collected blood samples were centrifuged at 900 × g for 15 min. Plasma levels of insulin, adiponectin, and fibroblast growth factor (FGF) 21 were measured using commercially-available enzyme linked immunosorbent assay (ELISA) kits according to the manufacturers' protocol (Insulin ELISA kit from Morinaga Institute of Biological Science, Tokyo, Japan; Mouse/rat adiponectin ELISA kit from Otsuka Pharmaceutical, Tokyo, Japan; FGF21 ELISA kit from R&D Systems Inc., Minneapolis, MN). Briefly, plasma samples were applied into the specific antibody-precoated microplate and subsequently reacted with the second antibody. The enzyme reaction yielded a color product and its colormetric intensity was measured using a microplate reader. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated using the following formula: fasting blood glucose (mg/dL) × fasting plasma insulin (mU/mL)/405. Hepatic total lipid was estimated using the method described by Folch et al. [29]. Triacylglycerol (TG) level in liver was measured using a commercial kit (Wako E-Test kit; Wako Pure Chemical Industries Ltd.).

2.6 Western blotting

Approximately 50 mg of liver tissues were homogenized by lysis buffer with a protease inhibitor cocktail (cOmplete^{TM} Mini; Roche, Mannheim, Germany). The homogenate

was centrifuged at 18000 × g for 30 min. The supernatant protein was obtained and determined by Bradford method using a Protein Assay Dye Regent Concentrate (Bio-Rad, Hercules, CA) with a commercial protein standard (bovine gamma globulin; Bio-Rad). The samples were separated by SDS-PAGE and transferred on to a polyvinylidene fluoride (PVDF) membrane (Trans-Blot® Turbo™ Transfer Pack and Sequi-Blot™ PVDF Membrane; Bio-Rad). After blockade with 5% skimmed milk for 1 h at room temperature, the membrane was incubated overnight with primary antibodies against AMP-activated protein kinase (AMPK) and phosphorylated-AMPK (p-AMPK) (Cell Signaling Technology Inc., Beverly, MA), fatty acid synthase (FAS) (Novus biologicals LLC, Littleton, CO), stearoyl-CoA desaturase (SCD)-1 (Santa Cruz Biotechnology Inc., Dallas, TX), acyl-CoA oxidase (AOX) (Proteintech Group Inc., Rosemont, IL), medium chain acyl-CoA dehydrogenase (MCAD) (Proteintech Group Inc.), and β-actin (Cell Signaling Technology, Inc.). The detail for primary antibody is described in Supplementary Table 2. Subsequently, the blotted membrane was washed and incubated with horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology Inc.), or anti-rabbit IgG (Cell Signaling Technology, Inc.) by recommended dilutions (1:2000). The detection of target protein was performed with chemiluminescence reagent (Clarity™ Western ECL Substrate; Bio-Rad).

2.7 Real-time polymerase chain reaction

Total RNA was isolated from liver, WAT, and BAT using TRIzol® reagent (Thermo Fisher Scientific Inc., Carlsbad, CA) according to the manufacturer's protocol. The RNA concentration was determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher). The RNA samples were diluted to 1 μg/μL and quantified using a real-time polymerase chain reaction (PCR) system (ABI PRISM 7500 Sequence Detection System; Applied Biosystems, Foster City, CA). Amplification was performed in a reacting solution containing 1 μg of RNA and specific primers with a QuantiTect SYBR Green Real-time PCR kit (Qiagen, Hilden, Germany). The primer sequences are listed in Supplementary Table 3. Thermal cycling conditions were as follows: 1 cycle of reverse transcription at 50°C for 30 min, initial activation at 95°C for 15 min, then 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. The expression levels were normalized to those of β-actin or 18s rRNA and analyzed using comparative C_T method. The gene expression levels are presented as ratio of experimental groups to Con group.

2.8 Statistical analysis

Data are presented as mean ± standard error. A significant difference ($P < 0.05$) between groups was assessed using one-way analysis of variance followed by Tukey Kramer post hoc tests for pair-wise comparisons. Values or groups sharing different letters are significantly different. All statistical analyses were performed using the Ekuseru-Toukei 2015 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

3. Results

3.1 The suppressive effect of fish oil on accumulation of subcutaneous fat was maintained after the discontinuation of fish oil

First, we discontinued the coadministration of fish oil with pioglitazone for 4 weeks and examined the effect on body weight, tissue weight, and abdominal fat mass in KK mice. Body weight and subcutaneous fat mass were significantly increased in P group compared to Con group (Table 1 and Fig. 1B). In fact, an obvious increase in body weight was observed from the 6^{th} week of pioglitazone treatment to the end of experimental period in P group (Fig. 2). However, these changes were significantly diminished by combination treatment with fish oil, which was maintained for 4 weeks after discontinuation of fish oil (Table 1 and Fig. 1B, 2). No significant between-group differences were observed with respect to epididymal WAT weight and visceral fat mass

(Table 1 and Fig. 1C). BAT weight was increased in pioglitazone-treated P, PF, and PF/P groups compared to Con group; however, the gain was significantly smaller in PF group among these groups (Table 1).

3.2 Discontinuation of fish oil did not affect the therapeutic efficacy of pioglitazone, but exhibited negative effects on adipocyte size and plasma adiponectin.

We performed biochemical assays, hepatic immunoblot and gene expression analyses, and morphological analysis of epididymal WAT to evaluate the effect of the discontinuation of fish oil after combination treatment with pioglitazone and fish oil. There were no significant differences in blood glucose levels among all groups. Plasma insulin level and HOMA-IR index significantly decreased in P group compared to Con group, and those in PF and PF/P groups were significantly lower to the same extent as in the P group (Table 2). The plasma adiponectin level in the P group was 2.56 times higher than that in the Con group, even the difference did not reach statistical significance (Table 2). The PF group showed significantly a higher plasma adiponectin level compared to Con and P groups; however, no significant increase in plasma adiponectin level was observed between the P and PF/P groups (Table 2). Adiponectin regulates insulin sensitivity in liver and skeletal muscle; it is secreted by small

adipocytes rather than hypertrophic adipocytes [30, 31]. Adipocyte size distribution revealed more frequent distribution of large adipocytes (6400–19600 μm^2) in Con group than in P group (Fig. 3A, 3B). Small adipocytes (0–900 μm^2) were more frequent in the P and PF groups compared to PF/P group, and the adipocytes (1600–4900 μm^2) in the PF and PF/P groups were more frequent than the P group (Fig. 3A, 3B). The mean adipocyte area in the P group was significantly smaller than that in the Con group. Furthermore, in the PF group, the mean adipocyte area was significantly smaller than that in the P group; however, there was no significant difference between the P and PF/P groups (Fig. 3A, 3C).

3.3 Pioglitazone and fish oil did not activate thermogenesis and energy expenditure, and pioglitazone dominates uptake of glucose than fatty acid in BAT

The uncoupling protein-1 (UCP1), one of the mitochondrial uncoupling proteins, is specific for BAT, and plays a key role in consumption of energy as heat [32]. The UCP1-mediated BAT thermogenesis contributes to whole-body energy expenditure; therefore, the activation of BAT regulates the body fat mass and has a protective effect against obesity and metabolic disorders [33]. Therefore, we focused on energy expenditure in BAT as a mechanism by which the discontinuation of fish oil after

combination treatment with pioglitazone and fish oil maintained the suppressive effect of fish oil on the accumulation of subcutaneous fat. In this study, we observed no significant between-group differences with respect to the mRNA levels of UCP1 and carnitine palmitoyltransferase-1 (CPT1), mitochondrial proteins involved in fatty acid β-oxidation (Fig. 3D). Glucose transporter (GLUT) 4 mRNA level tended to increase in P group ($p = 0.0618$) and was significantly higher in PF/P group than Con group. Conversely, gene expressions of CD36, a long-chain fatty acid transporter, were significantly decreased in P and PF/P groups compared with Con group. However, no significant differences were observed between the Con and PF groups with respect to levels of both GLUT4 and CD36 mRNA (Fig. 3D).

3.4 Pioglitazone treatment upregulated the expression of genes related to thermogenesis in WAT with or without fish oil; however, these changes subsided after the discontinuation of fish oil regardless of continuation of pioglitazone monotherapy

Next, we examined the effect of discontinuation of fish oil on other type of UCP1 expressing adipose tissue, including “beige adipocyte”, which is a potential target for anti-obesity interventions owing to its ability to metabolize glucose and oxidize lipids [34]. Induction of beige adipocytes in WAT is referred to as “WAT browning”, a process

which is promoted by PPARγ agonist and fish oil treatment [35, 36]. Interestingly, UCP1 mRNA level in WAT tended to increase in the PF group ($p = 0.0789$); in addition, its expression level in PF/P group was significantly higher than that in the Con group (Fig. 3E). Gene expressions of thermoregulatory markers [such as CIDEA, a CIDE (cell-death-inducing DNA-fragmentation-factor-45-like effector) family protein, and cytochrome c oxidase subunit 7a1 (Cox7a1)] in PF group were higher than those in the Con group ($p = 0.0697$ and 0.0689, respectively) (Fig. 3E). CPT1 mRNA level in the P and PF groups ($p = 0.0569$ and 0.0676) were higher than that in the Con group; however, no significant difference in this respect was observed between the Con and PF/P groups (Fig. 3E). We measured plasma and mRNA levels of FGF21, which is a hormonal factor that upregulates UCP1 expression in WAT and BAT. PF group showed the highest plasma levels and hepatic mRNA levels of FGF21 among all groups; however, the difference from the corresponding levels in the Con group was not statistically significant (Table 2 and Fig. 4D). There was also no significant the difference between the groups with respect to the change in FGF21 mRNA levels in WAT (Fig. 3E).

3.5 The activated β-oxidation and suppressed lipogenesis in liver by pioglitazone with fish oil disappeared after the discontinuation of fish oil

AMPK plays a critical role in the regulation of fatty acid and energy metabolism [37]. Moreover, the activation of hepatic AMPK helps improve type 2 diabetes via the downregulation of expressions of gluconeogenesis-related genes, such as glucose 6-phosphatase (G6pase) and PEPCK [38]. G6Pase catalyzes the hydrolysis of glucose-6-phosphate to glucose in the last step of glycogenolysis and gluconeogenesis [39]. PEPCK also plays an important role in glucose formation via conversion of oxaloacetate to phosphoenolpyruvate [40]. There were no significant between-group differences with respect to changes in protein expressions of p-AMPK, total AMPK (t-AMPK), and ratio of p-AMPK to t-AMPK in liver (Fig. 4A, 4B). Similarly, the hepatic gene expressions of G6pase and PEPCK remained unchanged among all groups (Fig. 4D).

Next, we investigated the mechanisms by which the suppressive effect on body weight gain and accumulation of subcutaneous fat was maintained after discontinuation of fish oil. In our previous study, the combination of pioglitazone and fish oil was found to stimulate fatty acid β-oxidation and inhibit de novo lipogenesis in liver [23]. Thus, we measured the hepatic expressions of AOX and MCAD as markers of peroxisomal and mitochondrial fatty acid β-oxidation, respectively. Protein expressions of AOX and MCAD were significantly elevated in PF group compared to Con group; however, there

were no significant differences in this respect between the Con, P, and PF/P groups (Fig. 4A, 4C). Although AOX mRNA level showed the same tendency as observed for AOX protein level, MCAD mRNA level in the PF and PF/P groups was significantly higher than that in the Con group (Fig. 4D). And, we evaluated hepatic de novo lipogenesis-related markers, such as FAS and SCD1. The protein expression of SCD1 in the P group was significantly higher than that in the Con group, while that in the PF group was significantly lower than that in the Con group; no significant difference in this respect was observed between Con and PF/P groups (Fig. 4A, 4C). Similarly, the protein expression of FAS tended to increase in P group ($p = 0.0948$) compared to Con group; however, there were no significant differences in this respect between Con, PF, and PF/P groups (Fig. 4A, 4C). And, no significant between-group differences were observed with respect to the gene expressions of FAS and SCD1 (Fig. 4D).

4. Discussion

Over 20 years ago, TZDs were shown to promote the differentiation of preadipocytes into mature adipocytes in subcutaneous fat, but not in visceral fat [41]. Several studies have shown that TZD-induced weight gain is associated with an increase in subcutaneous adipose tissue [42, 43]. In our recent study, combination therapy with

pioglitazone and fish oil for 8 weeks was found to ameliorate pioglitazone-induced subcutaneous fat accumulation and body weight gain via the suppression of lipogenesis and the activation of fatty acid β-oxidation in liver of young and aged KK mice [20, 23]. In agreement with our previous study, the present study confirmed the suppressive effect of fish oil on the pioglitazone-induced accumulation of subcutaneous fat and body weight gain. Importantly, these effects persisted for 4 weeks after discontinuation of fish oil along with an improvement in insulin resistance; however, the discontinuation of fish oil caused negative effects on lipid metabolism.

Our previous studies also found that the above-mentioned suppressive effect of fish oil was related to the inhibition of pioglitazone-induced fatty acid synthesis in liver [20, 23]. Although hepatic FAS and SCD1 gene expressions did not significantly change in pioglitazone and/or fish oil-treated mice, these protein expressions were increased in mice treated with pioglitazone monotherapy. Our results showed that fish oil treatment suppressed pioglitazone-induced upregulation of hepatic FAS and SCD1 protein levels, an effect that was abolished after the discontinuation of fish oil for 4 weeks.

Several researchers have reported an anti-obesity effect of n-3 PUFA that is mediated via activation of PPARα and increased fatty acid β-oxidation in liver [44-46]. Moreover, PPARα agonist was shown to prevent adipocyte hypertrophy in epididymal WAT and an

increased subcutaneous fat weight by PPARγ agonist [47]. In this study, combination therapy with fish oil and pioglitazone markedly upregulated hepatic PPARα-related gene and protein expressions, such as AOX and MCAD; this was associated with an increased plasma adiponectin level and a decrease in the size of adipocytes in epididymal WAT. Adiponectin secretion is due to the direct effect of PPARγ ligands and n-3 PUFA on the upregulation of mRNA level in adipocytes; in addition, small adipocytes are more active secretors of adiponectin than hypertrophic adipocytes [30, 48]. Therefore, the combination of pioglitazone and fish oil activated PPARα-mediated hepatic fatty acid β-oxidation and inhibited adipocyte hypertrophy, which contributed to the secretion of adiponectin, in addition to PPARγ-mediated effects on epididymal WAT. Since the discontinuation of fish oil for 4 weeks attenuated the activation of PPARα in liver and partially attenuated PPARγ in WAT, the antihypertrophic effect on adipocytes and adiponectin secretion were also canceled. This also means that the pioglitazone-induced accumulation of subcutaneous fat and body weight gain could be observed after more than 4 weeks of fish oil discontinuation.

On the other hand, an edema is more frequently seen as a side effect in patients treated with TZDs [49]. The body weight gain without an accumulation of visceral and subcutaneous fats was observed by discontinuation of fish oil, which suggested that the

water retention was related to the pioglitazone-induced body weight gain and suppressed, at least in part, by the combination of pioglitazone and fish oil.

One of the effects of adiponectin on liver and skeletal muscle is an improvement in insulin sensitivity and lipid metabolism by the activation of AMPK and PPARα through specific receptors for adiponectin (AdipoR1 and R2, respectively) [50, 51]. We had also shown that pioglitazone treatment reduced insulin resistance associated with increased plasma adiponectin level of more than 20 μg/mL in male KK mice [20, 23]. Consistent with these reports, pioglitazone-treated mice in the present study showed an increase in plasma adiponectin level and a decrease in HOMA-IR index. However, changes in the gene expressions of gluconeogenic enzyme associated with the activation of AMPK were not observed in the liver. Adiponectin was shown to promote glucose uptake and fatty acid β-oxidation in skeletal muscle through AdipoR1-mediated AMPK and p38 mitogen-activated protein kinase (MAPK) pathways [52, 53]. Moreover, PPARα transcriptional activity is stimulated by the adiponectin-induced activation of AMPK and MAPK [54]. In other studies, adiponectin itself also increases the expressions of PPARα and endogenous ligands in skeletal muscle [4, 55], which suggests that pioglitazone-induced adiponectin may potentially help improve glucose and fatty acid metabolism in skeletal muscle than liver. The effect of pioglitazone and fish oil on

skeletal muscle is not clear; therefore, further studies might be needed.

In addition to the direct activation of hepatic PPARα by fish oil, we focused on FGF21, a hormonal factor that is abundantly expressed in liver, but to a lesser extent in WAT and skeletal muscle [56]. FGF21 increases hepatic fatty acid β-oxidation via the induction of PPAR γ coactivator protein-1α and acts in an autocrine/paracrine manner to activate thermogenic pathways and induce browning of WAT [57]. In a recent study, PPARα agonist was shown to enhance hepatic FGF21 production and FGF21-WAT browning pathway [58]; and, Yang et al reported that 20 wt% fish oil feeding upregulated hepatic FGF21 mRNA level, but failed to increase serum FGF21 level and its protein expression in C57BL/6J mice [59]. In this study, 10 wt% fish oil-treated mice did not show any significant impact on plasma FGF21 level and its gene expressions in liver, which suggests that further doses of fish oil may be required to induce hepatic secretion of FGF21.

PPARγ agonists have been shown to induce WAT browning in primary adipocytes and animal models [60, 61]. As expected, pioglitazone and fish oil-treated mice showed increased expressions of UCP1 and thermoregulatory genes in epididymal WAT. Despite continuation of pioglitazone treatment, the discontinuation of fish oil markedly attenuated pioglitazone-induced upregulation of thermoregulatory genes up to the level

in the Con group, but not that of UCP1. These observations suggested that the disappearance of thermogenesis was in part a negative effect of discontinuation of fish oil.

Consistent with the previous study that used 3T3-L1 adipocyte with PPARγ ligand [62], pioglitazone monotherapy increased GLUT4 mRNA level in BAT. Although fish oil feeding upregulated PPARγ and GLUT4 expressions in BAT [63], pioglitazone-induced upregulation of GLUT4 mRNA was completely canceled in fish oil-treated mice in our study. On the other hand, pioglitazone treatment decreased CD36 mRNA level in BAT, and its downregulation was also attenuated in the presence of fish oil. The underlying mechanisms are yet to be elucidated; however, the phenomenon of brown-to-white conversion in adipose tissue (“BAT whitening”) is a potential explanation [64]. BAT mass was increased by lipid accumulation in obesity condition, which creates WAT-like phenotype and declines typical BAT function, such as thermogenesis, lipolysis, glucose, and fatty acid uptake [65]. In the present study, BAT mass was greater in mice treated with pioglitazone without fish oil and it acquired WAT-like color (own observation), which confirms that this was related to lipid accumulation in BAT. These observations were normalized in fish oil-treated mice, which suggest that fish oil cotreatment improved pioglitazone-induced BAT whitening and had an effect on some gene

expressions. PPARγ agonist-induced BAT whitening in KK mice is still a matter of debate. Future trials should focus on BAT dysfunction as a therapeutic strategy for type 2 diabetes using pioglitazone and fish oil.

In conclusion, in the present study, the suppressive effect of fish oil on pioglitazone-induced accumulation of subcutaneous fat and body weight gain was maintained in spite of discontinuation of fish oil cotreatment for 4 weeks in KK mice. However, the discontinuation of fish oil showed several negative effects: 1) return of plasma adiponectin level; 2) abolition of inhibition of lipogenesis and activation of fatty acid β-oxidation in liver; 3) increase in hypertrophic adipocytes in epididymal WAT; and 4) accumulation of lipids in BAT. Insulin resistance was improved by pioglitazone with or without fish oil treatment. These findings indicate that continued treatment of TZDs and n-3 PUFA produces the desired therapeutic effect, and n-3 PUFA interruption for at least 4 weeks causes adverse effects on lipid metabolism and energy homeostasis.

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Conflict of interest

The authors declare that they have no competing financial interests.

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Legends for figures

Supplementary Figure 1 Experimental design

Figure 1 Impact of discontinuation of fish oil after pioglitazone-fish oil combination treatment on abdominal fats.

CT images (A), subcutaneous fat mass (B), and visceral fat mass (C). Colored regions represent subcutaneous fat (yellow) and visceral fat (pink). The data are expressed as mean ± SE (n = 4-5), and analyzed by one-way ANOVA followed by the Tukey Kramer post hoc tests. Groups sharing different letters are significantly different at $P < 0.05$.

Figure 2 Impact of discontinuation of fish oil after pioglitazone-fish oil combination treatment on body weight.

Body weight was measured weekly. Measurement of body weight in 12 weeks was only performed under 12 h-fasted condition for dissection. Data are presented as mean ± SE (n = 5), and analyzed by one-way ANOVA followed by the Tukey Kramer post hoc tests. *Significant difference between Con and P groups at $P < 0.05$; ** Significant difference between P and PF groups at $P < 0.05$; # Significant difference between P and PF/P groups at $P < 0.05$; ## Significant difference between Con and PF/P groups at $P < 0.05$.

Figure 3 Impact of discontinuation of fish oil after pioglitazone-fish oil combination treatment on adipocyte size of WAT and gene expressions in BAT and WAT.

Sections of WAT stained with hematoxylin-eosin(A), adipocyte distribution of WAT (B), mean adipocyte area of WAT (C), mRNA expression levels in BAT (D) and WAT (E). The results of gene expression are represented as ratio of experimental groups to Con group after normalization by the 18s rRNA (BAT) or β-actin (WAT). The data are expressed as mean ± SE (n = 4-5), and analyzed by one-way ANOVA followed by the Tukey Kramer post hoc tests. Groups sharing different letters are significantly different at $P < 0.05$. Abbreviations: Ucp1, uncoupling protein 1; Glut4, glucose transporter 4; Cd36, fatty acid transporter CD36; Cpt1, carnitine palmitoyl transferase 1; Cidea, cell-death-inducing DNA-fragmentation-factor-45-like effector; Cox7a1, cytochrome c oxidase subunit 7a1; Fgf21, fibroblast growth factor 21.

Figure 4 Impact of discontinuation of fish oil after pioglitazone-fish oil combination treatment on hepatic protein and gene expressions.

Western blot analysis of hepatic protein expressions (A), Quantitative values of protein levels in liver (B), (C), and mRNA expression levels in liver (D). The results are represented as ratio of experimental groups to Con group after normalization by the

β-actin. The data are expressed as mean ± SE (n = 4-5), and analyzed by one-way ANOVA followed by the Tukey Kramer post hoc tests. Groups sharing different letters are significantly different at $P < 0.05$. Abbreviations: Fas, fatty acid synthase; Scd1, stearoyl-CoA desaturase 1; Aox, acyl-CoA oxidase; Mcad, medium-chain acyl-CoA dehydrogenase; Fgf21, fibroblast growth factor 21; G6pase, glucose 6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase.

Supplementary Figure 1

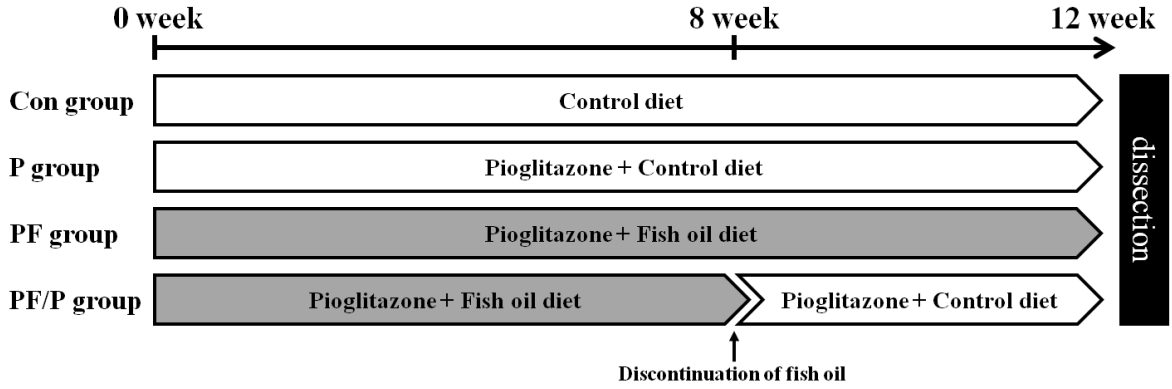


Figure 1

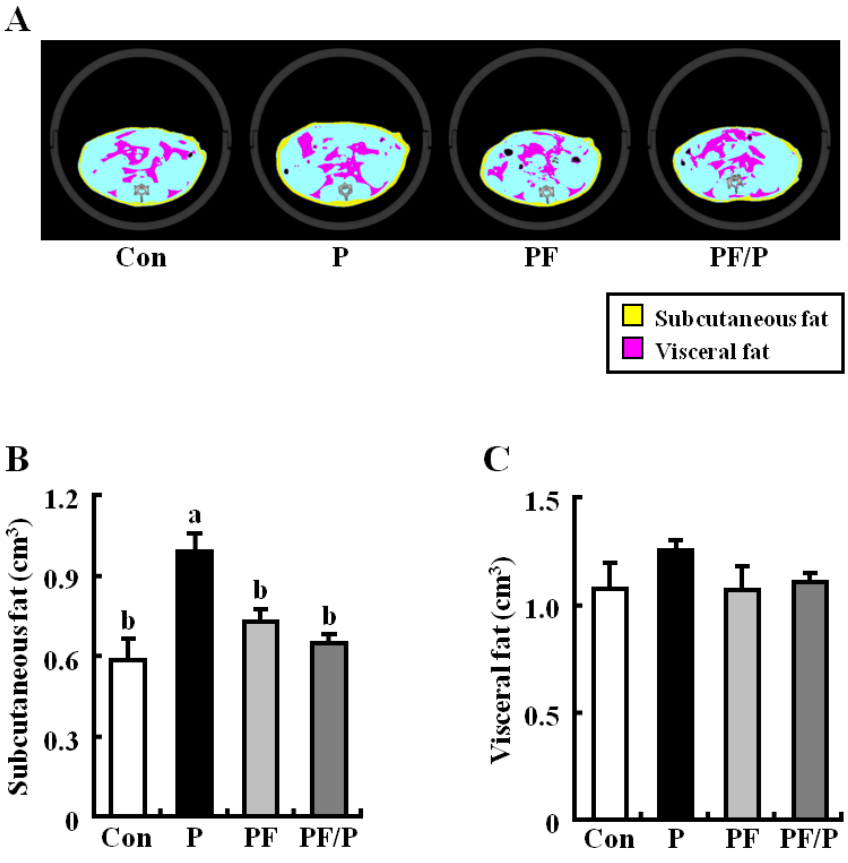


Figure 2

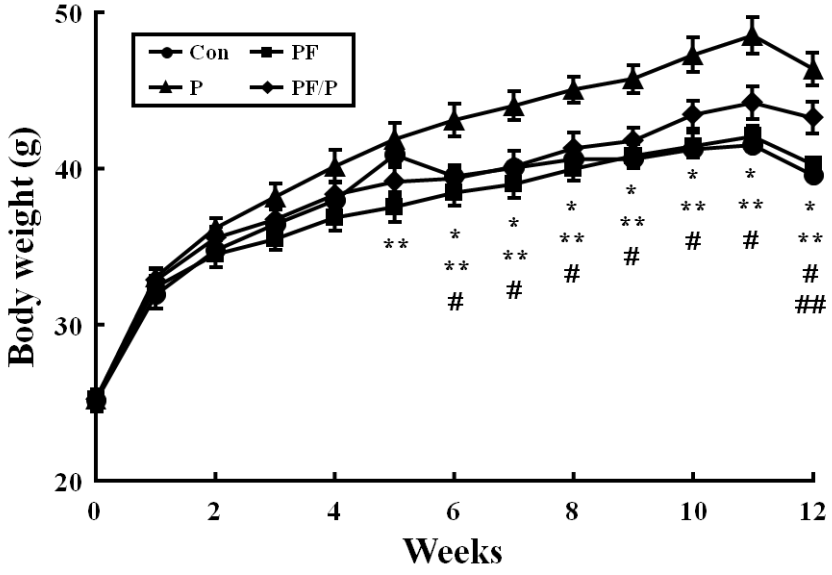
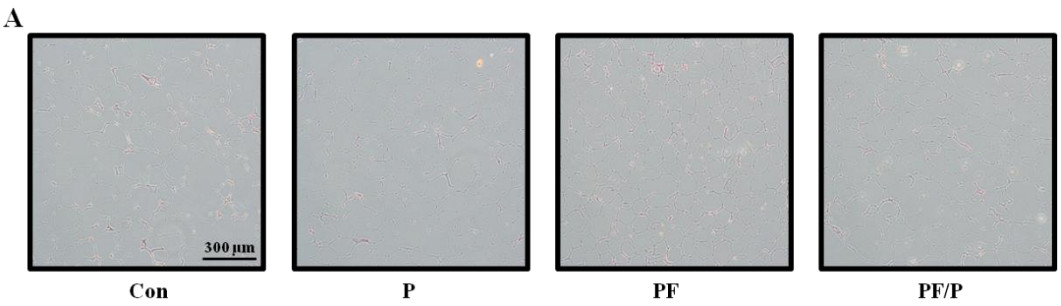


Figure 3



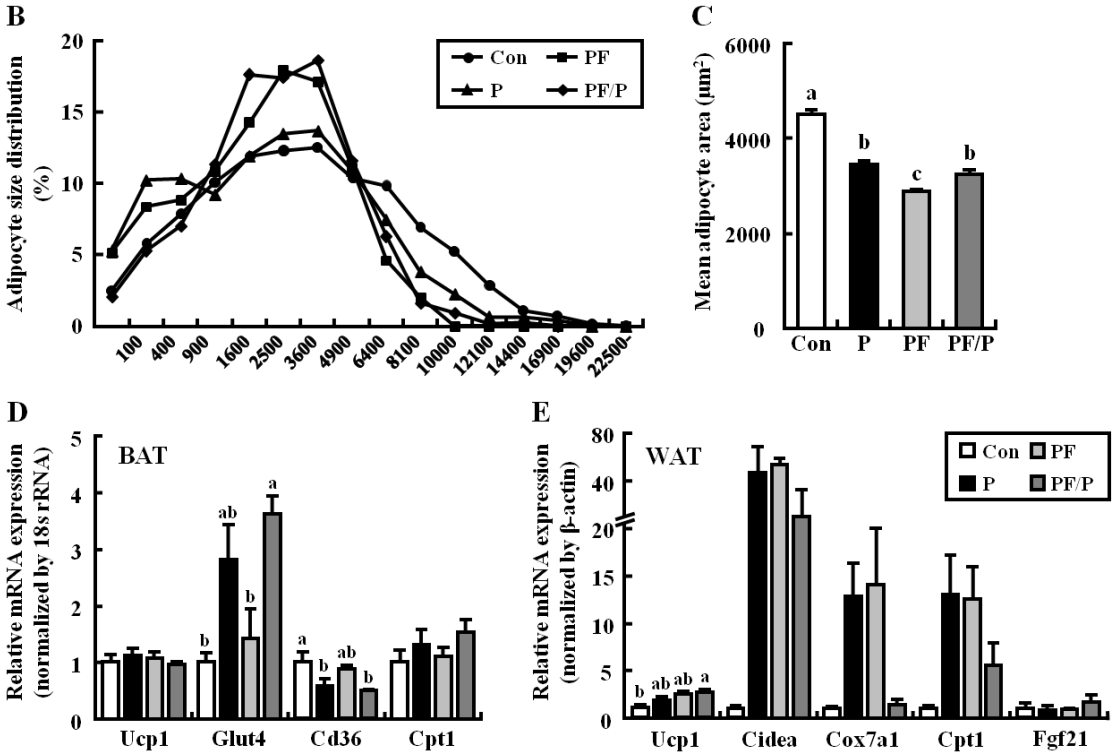
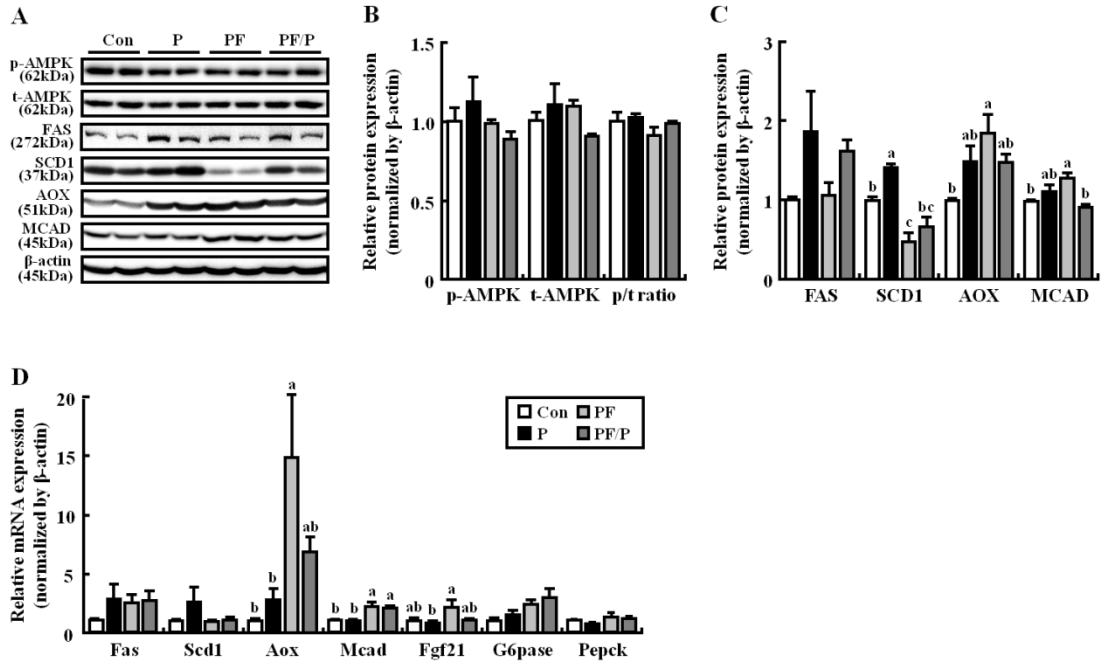

B
Adipocyte size distribution (%)
Con
PF
P
PF/P
100
400
900
1600
2500
3600
4900
6400
8100
10000
12100
14400
16900
19600
22500-
C
Mean adipocyte area (μm²)
Con
P
PF
PF/P
D
BAT
Relative mRNA expression (normalized by 18s rRNA)
Ucp1
Glut4
Cd36
Cpt1
E
WAT
Relative mRNA expression (normalized by β-actin)
Ucp1
Cidea
Cox7a1
Cpt1
Fgf21

Figure 4


A
Con
P
PF
PF/P
p-AMPK (62kDa)
t-AMPK (62kDa)
FAS (272kDa)
SCD1 (37kDa)
AOX (51kDa)
MCAD (45kDa)
β-actin (45kDa)
B
Relative protein expression (normalized by β-actin)
p-AMPK
t-AMPK
p/t ratio
C
Relative protein expression (normalized by β-actin)
FAS
SCD1
AOX
MCAD
D
Relative mRNA expression (normalized by β-actin)
Fas
Scd1
Aox
Mcad
Fgf21
G6pase
Pepck

Supplementary Table 1. Composition of experimental diets.

Group	Control diet	fish oil diet
Safflower oil (g)	8	4
Fish oil (g)	-	4
Casein (g)	20	20
Sucrose (g)	10.37	10.37
β-starch (g)	51.83	51.83
Vitamin mix* (g)	1	1
Mineral mix* (g)	3.5	3.5
Cellulose powder (g)	5	5
L-cystin (g)	0.3	0.3
t-Butylhydroquinone (g)	0.0016	0.0016
Total (g)	100.00	100.01
Energy (kcal/100g)	374.02	373.99
Fat energy (%)	19.70	19.70

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

Supplementary Table 2. Condition of primary antibody reactions in western blotting analysis.

Target protein	Host animal	Dillution	Incubeted condition
AMPK	Rabbit	1:2000	16 hour, 4°C
p-AMPK	Rabbit	1:1000	16 hour, 4°C
FAS	Rabbit	1:1000	16 hour, 4°C
SCD-1	Goat	1:500	16 hour, 4°C
AOX	Rabbit	1:1000	16 hour, 4°C
MCAD	Rabbit	1:1000	16 hour, 4°C
β-actin	Rabbit	1:1000	16 hour, 4°C

Supplementary Table 3. Primer sequences for real-time PCR.

Genes	Forward primer (5'-3')	Reverse primer (3'-5')
Fas	tcaccactgtgggctctgcagagaagcgag	tgtcattggcctcctcaaaaagggcgtcca
Scd1	ccggagacccttagatcga	tagcctgtaaaagatttctgcaaacc
Aox	tcaacagcccaactgtgacttccatta	tcaggtagccattatccatctcttca
Mcad	agggtttagttttgagttgacgg	ccccgcttttgtcatattccg
Fgf21	gctgctggaggacggttaca	cacaggtccccaggatgttg
G6pase	actgtgggcatcaatctcctc	cgggacagacagacgttcagc
Pepck	gtgctggagtggatgttcgg	ctggctgattctctgtttcagg
Ucp1	ggaggtgtggcagtgttcattgg	agcattgtaggtccccgtgtagcg
Cidea	aatggacaccgggtagtaagt	cagcctgtataggtcgaaggt
Cpt1	ccaggctacagtgggacatt	gaacttgcccatgtccttgt
Cox7a1	aaaaccgtgtggcagagaag	ccagcccaagcagtataagc
Glut4	tactcattcttggacggttcctc	ggagtactgtgagagccagaagc
Cd36	gcaaaacgactgcaggtcaac	tggtcccagtctcatttagcca
β-actin	catcctggcctcactgtcca	agtacgatgagtccggcccc
18s rRNA	ccatccaatcggtagtagcg	gtaacccgttgaaccccatt

Table 1. Total food intake, body weight, and tissue weight in 19-week-old male KK mice.

Group	Con	P	PF	PF/P
Total food intake (g/mouse)	401 ± 12^{a}	375 ± 11^{ab}	341 ± 4^{b}	355 ± 10^{b}
Initial body weight (g)	25.2 ± 0.7	25.2 ± 0.5	25.2 ± 0.5	25.2 ± 0.3
Final body weight (g)	39.6 ± 0.2^{c}	46.4 ± 1.0^{a}	40.2 ± 0.5^{bc}	43.3 ± 1.0^{b}
Liver weight (g)	1.88 ± 0.12^{ab}	2.11 ± 0.16^{a}	1.58 ± 0.08^{b}	1.75 ± 0.12^{ab}
Epididymal WAT weight (g)	1.05 ± 0.10	0.89 ± 0.07	0.87 ± 0.03	0.83 ± 0.02
BAT weight (g)	0.21 ± 0.01^{c}	1.08 ± 0.11^{a}	0.69 ± 0.12^{b}	1.18 ± 0.08^{a}

Data are represented as mean ± SE (n = 5). Values sharing different superscripts in a same row are significantly different: $P < 0.05$ by Tukey–Kramer test.

Table 2. Biochemical parameter in 19-week-old KK male mice.

Group	Con	P	PF	PF/P
Blood glucose (mg/dl)	181 ± 21	236 ± 34	198 ± 34	209 ± 20
Plasma insulin (ng/ml)	17.8 ± 2.9^{a}	5.7 ± 1.3^{b}	3.0 ± 0.6^{b}	2.0 ± 0.9^{b}
HOMA-IR	1.00 ± 0.20^{a}	0.39 ± 0.09^{b}	0.19 ± 0.06^{b}	0.13 ± 0.07^{b}
Plasma adiponectin (μg/ml)	7.5 ± 2.9^{c}	19.2 ± 1.1^{bc}	40.5 ± 5.1^{a}	24.9 ± 3.1^{b}
Plasma FGF21(ng/ml)	4.02 ± 0.90	4.34 ± 1.03	5.95 ± 0.97	4.20 ± 0.89
Hepatic TG (mg/g liver)	103 ± 20	175 ± 41	87 ± 25	119 ± 28

Data are represented as mean ± SE (n = 4-5). Values sharing different superscripts in a same row are significantly different: $P < 0.05$ by Tukey–Kramer test.