Studies on the attenuation effects of intestinal PPARα activation on postprandial hyperlipidemia

Rino Kimura

2014

Contents

General Introduction

A great deal of change in diet and lifestyle has occurred these last decades in most areas of the world, among which significant increase in caloric intake and decrease in exercise (1). The alterations in triglyceride (TG) and lipoprotein metabolism are transitory and usually last from 6 to 8 h after ingestion of a fatty meal in human (2). Due to frequent ingestion of such meal, individuals spend the majority of the day in the fed state. The state of rapid increase in plasma TG levels or the prolonged state of high plasma TG levels after fatty meal ingestion is called “postprandial hyperlipidemia”. Several changes as above have occurred simultaneously with a rise in metabolic diseases, such as obesity, metabolic syndrome, and diabetes, that are risk factors of atherosclerosis and cardiovascular diseases. Therefore, many studies have suggested that the development of these pathologies could be associated with postprandial hyperlipidemia (3-5), even in patients without fasting hyperlipidemia (6).

Since Zilversmit first proposed that the postprandial chylomicron is the most common risk factor for atherogenesis over 30 years ago (7), several studies have shown that TG-rich lipoproteins (TRLs) and their remnants are significantly increased in postprandial plasma and are known to predict the risk of cardiovascular disease (CVD) (8, 9), independent of the total cholesterol, LDL or HDL cholesterol level. Recently, non-fasting TG levels have come to be known as a significant risk indicator for CVD events (10, 11). Iso, H. et al have reported that non-fasting serum TGs predict the incidence of coronary heart disease among Japanese men and women who possess low mean values of total cholesterol (12). Additionally, numerous prospective case-control studies have qualitatively established postprandial TG as a risk factor for CVD (13).

Dramatic postprandial hyperlipidemia is frequently associated with a number of impaired metabolic processes, including changes of activity of enzymes involved in processing of TRLs, uptake of TRLs and remnants by peripheral tissue, and synthesis and secretion of lipoproteins from the small intestine and the liver. These abnormalities are well recognized to be disturbed by genetic factors, obesity, and insulin resistance (14). In addition, it is also well known that the postprandial TG response is influenced by both the amount and type of dietary fat given in a meal. Dietary and lifestyle modifications are thought to be the cornerstone of clinical management of postprandial hyperlipidemia. A combination of diet modification and drug therapy may also be considered to be the first-line lipid-regulating therapy on the basis of the well-characterized efficacy and safety profiles of it. However, it is noteworthy that there are no existing specific therapeutic targets for postprandial hyperlipidemia. Therefore, elucidation of specific therapeutic targets for it and screening of food-derived effective compounds are needed.

After a fatty meal is ingested, the small intestine is directly exposed to dietary TGs, which are hydrolyzed by the enzyme pancreatic lipase to free fatty acids and monoglycerides. They are then absorbed in intestinal epithelial cells and transported to the endoplasmic reticulum for resynthesis of TGs. Chylomicron assembled with resynthesized TG and apolipoprotein (apo) B-48 is secreted into the circulation via lymph vessels. ApoB-48 is the constituent protein of chylomicron and reflects the number of circulating particles of chylomicrons and their remnants (15). Then, chylomicron is hydrolyzed by lipoprotein lipase (LPL) producing a chylomicron remnant particle, and is in turn cleared by the liver. Thus, the small intestine plays an important role in uptake of fatty acids into the body. Therefore, reduction in

chylomicron secretion via the control of lipid metabolism in intestinal epithelial cells could improve postprandial hyperlipidemia.

This study aimed to elucidate whether PPARα activation affects lipid metabolism in intestinal epithelial cells, which attenuates postprandial hyperlipidemia. In chapter 1, it was revealed that PPARα activation induces fatty acid oxidation in intestinal epithelial cells, which attenuates postprandial hyperlipidemia. In chapter 2, it was found that intestinal PPARα activation increases fatty acid oxidation and reduces postprandial hyperlipidemia in obese diabetic condition. In chapter 3, it was elucidated that docosahexaenoic acid (DHA) enhances fatty acid oxidation in intestinal epithelial cells, which attenuates postprandial hyperlipidemia via PPARα activation.

REFERENCES

1. Hernández Vallejo SJ, Alqub M, Luquet S, Cruciani-Guglielmacci C, Delerive P, Lobaccaro JM, Kalopissis AD, Chambaz J, Rousset M, Lacorte JM. 2009. Short-term adaptation of postprandial lipoprotein secretion and intestinal gene expression to a high-fat diet. *Am J Physiol Gastrointest Liver Physiol.* 296(4):G782-92.
2. Chan DC, Pang J, Romic G, Watts GF. 2013. Postprandial hypertriglyceridemia and cardiovascular disease: current and future therapies. *Curr Atheroscler Rep.* 15:309.
3. Grønholdt M, Nordestgaard B, Nielsen T, Sillesen H. 1996. Echolucent carotid artery plaques are associated with elevated levels of fasting and postprandial triglyceride-rich lipoproteins. *Stroke* 27: 2166–2172.
4. Karpe F, Steiner G, Uffelman K, Olivecrona T, Hamsten A. 1994. Postprandial

lipoproteins and progression of coronary atherosclerosis. *Atherosclerosis.* 106: 83–97.

5. Patsch J, Miesenbo¨ck G, Hopferwieser T, Mu¨hlberger V, Knapp E, Dunn J, Gotto AJ, Patsch W. 1992. Relation of triglyceride metabolism and coronary artery disease. Studies in the postprandial state. *Arterioscler Thromb.* 12: 1336–1345.
6. Groot P, van Stiphout W, Krauss X, Jansen H, van Tol A, van Ramshorst E, Chin-On S, Hofman A, Cresswell S, Havekes L. 1991. Postprandial lipoprotein metabolism in normolipidemic men with and without coronary artery disease. *Arterioscler Thromb.* 11: 653–662.
7. Zilversmit DB. 1979. Atherogenesis: a postprandial phenomenon. *Circulation.* 60:473-85.
8. Havel RJ. 1994. Postprandial hyperlipidemia and remnant lipoproteins. *Curr Opin Lipidol.* 5:102–9.
9. Havel RJ. 2000. Remnant lipoproteins as therapeutic targets. *Curr Opin Lipidol.* 11: 615–20
10. Nordestgaard BG, Benn M, Schnohr P, Tybjaerg-Hansen A. 2007. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. *JAMA.* 298:299–308.
11. Bansal S, Buring J, Rifai N, Mora S, Sacks FM, Rider PM. 2007. Fasting compared with nonfasting triglycerides and risk of cardiovascular events in women. *JAMA.* 298:309–16.
12. Iso H, Naito Y, Sato S, Kitamura A, Okamura T, Sankai T, Shimamoto T, Iida M, Komachi Y. 2001. Serum triglycerides and risk of coronary heart disease among Japanese men and women. *Am J Epidemiol.* 153:490–9.

13. Lopez-Miranda J, Williams C, Lairon D. 2007. Dietary, physiological, genetic and pathological influences on postprandial lipid metabolism. *Br J Nutr*;98:458–73.
14. Jackson KG, Poppitt SD, Minihane AM. 2012. Postprandial lipemia and cardiovascular disease risk: Interrelationships between dietary, physiological and genetic determinants. *Atherosclerosis*. 220:22-33.
15. Smith D, Watts GF, Dane-Stewart C, Mamo JC. 1999. Post-prandial chylomicron response may be predicted by a single measurement of plasma apolipoprotein B48 in the fasting state. *Eur J Clin Investig*. 29:204–9.

Chapter 1

Studies on effects of PPARα activation on intestinal lipid metabolism and postprandial hyperlipidemia

INTRODUCTION

Peroxisome proliferator-activated receptor (PPAR)-α is involved in regulation of lipid metabolism including fatty acid oxidation in various peripheral tissues such as the liver and skeletal muscle (1). PPARα is among nuclear receptors that are ligand-dependent transcriptional factors inducing mRNA expression of target genes (2). PPARα activation enhances fatty acid oxidation by inducing mRNA expression of fatty acid oxidation-related genes such as *acyl-CoA synthetase (Acs)*, *carnitine-palmitoyl-transferase-1a* (*Cpt1a*), and *acyl-CoA oxidase (Aox)* (2, 3). The PPARα-dependent enhancement of fatty acid oxidation decreases the levels of circulating and accumulated lipids. This is why synthetic PPARα agonists such as fibrates have been widely used as anti-hyperlipidemic drugs (4). We have identified and analyzed food-derived compounds that activate PPARα (5-8). These compounds decrease the amounts of lipids accumulated in hepatocytes *in vitro* and prevent development of fatty liver *in vivo*. Moreover, we have reported that fatty acid oxidation in adipocytes is induced by PPARα activation, thereby reducing lipid accumulation in adipocytes (9). Therefore, PPARα activation is considered to be effective for prevention and improvement of dyslipidemia.

High serum lipid levels under fasting conditions have been considered a risk of

cardiovascular diseases (10). However, many studies have revealed that serum lipid levels under postprandial conditions, rather than under fasting conditions, strongly correlate with the risk of cardiovascular diseases (11). After absorption of triglycerides (TGs) in intestinal epithelial cells, resynthesized TGs are assembled into chylomicrons together with apolipoprotein B (apoB), which is the main apolipoprotein of chylomicrons and reflects the number of circulating particles of chylomicrons and their remnants (12). The chylomicrons assembled in intestinal epithelial cells are transported into the circulation via the lymphatics (13). Therefore, regulation of intestinal lipid metabolism could prevent the unusual increase in plasma lipid levels in postprandial state.

In this study, we examined whether PPARα activation in intestinal epithelial cells could enhance intestinal fatty acid oxidation, which attenuates postprandial hyperlipidemia. Treatment with Bezafibrate, a synthetic PPARα agonist, increased the mRNA expression levels of fatty acid oxidation-related genes and oxygen consumption rate (OCR), and decreased TG secretion into the basolateral side in Caco-2 cells. Moreover, administration of Bezafibrate also enhanced fatty acid oxidation in intestinal epithelial cells and attenuated postprandial hyperlipidemia via PPARα activation in high-fat diet (HFD)-fed mice. These findings indicate that PPARα activation in intestinal epithelial cells results in attenuation of postprandial hyperlipidemia via enhancement of fatty acid oxidation by PPARα activation, suggesting that intestinal fatty acid oxidation is a novel target of PPARα treatment for prevention and improvement of hyperlipidemia.

MATERIALS AND METHODS

Chemicals and cell culture

Bezafibrate was purchased from Sigma (MO, USA) and dissolved in DMSO as a stock solution. All other reagents were from Sigma or Nacalai Tesque (Kyoto, Japan) and were guaranteed to be of reagent or tissue- culture grade.

Human Caco-2 cells were purchased from American Type Culture Collection (ATCC) and cultured in DMEM (100 mg/dL glucose) containing 10% FBS, 1% nonessential amino acid solution, and 10 mg/mL penicillin/streptomycin at 37 °C in 5% CO_2/95% air under a humidified condition. After seeding, Caco-2 cells were seeded at a density of 1.12×10^6 cells/mL on 12-well Transwell® plates (Corning Inc., MA, USA) for 2 weeks for differentiation into intestinal epithelial-like cells. For the evaluation of differentiation of Caco-2 cells, we measured intestinal alkaline phosphatase activity and transepithelial electrical resistance (TER). There was no significant change in these differentiation markers in all experiments (data not shown). Twenty-four hours before starting the experiments, apical medium was changed to DMEM containing 50 μM Bezafibrate, 600 μM taurocholic acid Na salt hydrates, and 500 μM oleic acid. At the same time, basolateral medium was also changed to serum-free DMEM. For apoB and TG measurements, basolateral medium was collected.

Animal experiments

Nine-week-old male C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). The mice were maintained under a constant 12-h light/dark cycle. The mice were maintained for 1 week on a standard diet and then divided into two groups with the same average body weight and plasma TG level. Each group was maintained on

HFD consisting of 60% (kcal%) fat or HFD containing 0.2% (w/w) Bezafibrate for 1 week. The energy intake of all the mice was adjusted by pair feeding. Thus, the levels of food intake of each group were almost the same (average food intakes were 2.88 ± 0.18 and 2.60 ± 0.17 g/day in the control HFD-fed and 0.2% Bezafibrate-fed mice, respectively).

To clarify whether the effects of Bezafibrate on intestinal lipid metabolism and postprandial hyperlipidemia involves PPARα activation, we used PPARα$^{-/-}$ mice with a C57BL/6 genetic background. PPARα$^{-/-}$ mice were fed HFD for 1 week, and were then divided into two groups with the same average serum TG level and body weight after 16 h fasting. Ten-week-old male PPARα$^{-/-}$ mice were maintained for 1 week either on HFD or HFD containing 0.2% Bezafibrate.

For measurement of gene expression using real-time quantitative RT-PCR, proximal 1/4 of the intestine was harvested from mice. After washing twice with cold PBS, intestinal epithelial cells were collected with a slide glass. The collected cells were stored in RNAlater (Ambion/Applied Biosystems, TX, USA) at −80 °C until use.

For measurement of fatty acid oxidation, intestinal epithelial cells were collected from the proximal 1/4 of the intestine and incubated in 1 mg/mL collagenase IA/HBSS for 40 min. The collected intestinal epithelial cells were washed with 1% FBS/DMEM three times and used for experiments.

For measurement of plasma TG concentration, blood samples were collected from the tail vein of non-anesthetized mice. Anesthesia was induced using sevoflurane in all experiments.

Gene expression

Total RNA samples were prepared from Caco-2 cells and intestinal epithelial cells using Sepasol Super-I (Nacalai Tesque) and an SV total RNA isolation system (Promega, WI, USA), respectively, as previously described (14). To quantify mRNA expression, PCR was performed using a fluorescence temperature cycler (LightCycler System: Roche Diagnostics, Mannheim, Germany), as described previously (15). Primer sets were designed using a PCR primer selection program at the web site of the Virtual Genomic Center from the GenBank database and the sequences were described in our previous reports (5, 6). Primers used in this experiment are listed in Table 1. To compare mRNA expression level among samples, the copy number of each transcript was divided by that of *36B4* showing a constant expression level. All mRNA expression levels are presented as the percentage of the control in each experiment.

Table 1 Primers used for quantitative real-time PCR

Gene	Forward primer	Reverse primer
human *36b4*	AAACTGCTGCCTCATATCCGG	TTGTAGATGCTGCCATTGTCGA
human *Acs*	CTGCTGTTTTCGCTGGGTCC	AGCAGAGCTTCGCAGCGGC
human *Cpt1a*	AGGCAGAAGAGGTGACGATCG	AATCATCAAGAAATGTCGCACGA
human *Aox*	AAGCAACAGCATCTGAGCGAAT	GGCATGGTGTCCTATTTGAACG
mouse *36b4*	CTTTGGCGGGATTAGTCGAAG	TGTGTGTCTGCAGATCGGGTAC
mouse *Acs*	GAGATATTCTGGCCACCGATCA	ACGTATCCCTGGACTAGGACCG
mouse *Cpt1a*	GGCCTCTGTGGTACACGACAA	CTCAGTGGGAGCGACTCTTCA
mouse *Aox*	CAGGATCCGACTGTTTACC	CTTGTTCGCGCAAGTGAGG

Oxygen consumption rate in Caco-2 cells

Oxygen consumption rate (OCR) indicative of mitochondrial respiration was determined using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience, MA, USA). The XF24 device created a transient 7 μl chambers above target cells in which

cells were monitored in real-time as previously reported (16). Caco-2 cells were cultured for 2 weeks on the customized Seahorse 24-well plates (Seahorse Bioscience, MA, USA). Differentiated Caco-2 cells were incubated in pre-warmed XF24 assay medium for 1 h. The assay media consisted of DMEM supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 1.9 g/L NaCl, and 25 mM glucose was added to each well. After equilibration for 30 min, 2-min measurements were performed at 3-min intervals with inter-measurement mixing to homogenize the oxygen in the medium. OCR (pmol/min) was divided by protein amount in each well.

Fatty acid oxidation in intestinal epithelial cells

The intestinal epithelial cells harvested from the control or HFD containing Bezafibrate-fed mice were incubated in DMEM containing 200 μM palmitic acid, 0.1% fatty-acid-free BSA, 200 μM l-carnitine, and [^{14}C] palmitic acid (1 μCi) (American Radiolabeled Chemicals, MO, USA) for 2 h. Fatty acid oxidation products were assessed as previously described (9) with modification. Briefly, the labeling medium containing intestinal epithelial cells was transferred to a 50-mL polypropylene tube. An uncapped 2-mL sample tube containing a piece of filter paper soaked in 3 N NaOH was placed inside a 50-mL sample tube. After the tube was sealed, 200 μl of 12 N HCl was added to the medium sample to release [^{14}C]-CO_2. The tube was then incubated at 37 °C for 24 h. The saturated filter paper containing trapped [^{14}C]-CO_2 was assessed for radioactivity in a liquid scintillation counter (LS6500, Beckman Coulter, CA, USA). The acidified medium was centrifuged and 200 μL of supernatant was assessed for the amount of [^{14}C]-labeled acid soluble metabolites, which include labeled ketone bodies. Protein concentration was determined using a Protein Assay kit (Bio-Rad, CA, USA).

Western blotting

Western blotting was carried out as previously described (14, 15). Briefly, proteins samples extracted from intestinal epithelial cells were subjected to SDS-PAGE on a 10% gel. Separated proteins were transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, Billerica, MA, USA), which were blocked with 5% non-fat dried milk in phosphate-buffered saline (PBS). The membranes were incubated with anti-mouse AOX antibody (Abcam, MA, USA) and anti-mouse β-actin (Cell Signaling Technology, MA, USA), respectively, and then with peroxidase-conjugated anti-rabbit IgG antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Protein bands were detected by chemiluminescence using an enhanced chemiluminescence (ECL) system (NEN Lifescience Products) in accordance with the manufacturer's instructions. The bands were quantitatively evaluated using National Institutes of Health (NIH) Image J software.

TG concentration in Caco-2 cells and in mice

To measure postprandial plasma TG and TG secretion from intestinal epithelial cells in mice, mice were injected without or with 0.5g/kg body weight Tyloxapol (T0307, Sigma) into the intraperitoneal cavity to block serum lipase activity, respectively. After 30 min, mice were administered an oral gavage of 300 μl olive oil and plasma collected up to four hours post-gavage. For measurement of secreted TG levels in Caco-2 cells and plasma TG levels in mice, we used triglyceride E Test WAKO (Wako, Osaka, Japan).

ApoB secretion in Caco-2 cells and in mice

For measurement of apoB amount in Caco-2 cells, ELISA was performed using an anti-human low-density lipoprotein APO-B antibody (Clone 12G10; Monosan, Uden, Netherland), affinity purified anti-apolipoprotein B (Rockland, PA, USA), and HRP-conjugated anti-goat IgG (Promega) as the capture, primary, and secondary antibodies, respectively. Details of procedures were previously described (17). HRP activity was detected using TMB peroxidase substrate (KPL, MD, USA). Purified human very low-density lipoprotein (VLDL; Chemicon Millipore, MA, USA) was used as the standard protein.

To measure postprandial plasma apoB48 in mice, western blotting was carried out as above. Briefly, plasma was mixed with Laemmli Sample buffer (Bio-Rad) (1:8) and boiled for 5 min at 95°C. Plasma samples were subjected to SDS-PAGE on a 5% gel, and were transferred to PVDF membranes, which were blocked with 5% non-fat dried milk in PBS. The membranes were incubated with anti-mouse apoB48/100 antibodies (Meridian Life Science, Memphis, TN, USA), and then with peroxidase-conjugated anti-rabbit IgG antibodies.

Statistical analysis

The data were presented as means ± S.E.M. and statistically analyzed using one-way ANOVA when their variances were heterogeneous and unpaired t-test. Differences were considered significant at $P < 0.05$.

RESULTS

Bezafibrate increased fatty acid oxidation and decreases TG and apoB secretion in Caco-2 cells

To examine the effects of PPARα activation on intestinal epithelial cells, we first performed in *in vitro* experiments using Caco-2 cells. Differentiated Caco-2 cells treated with 50 μM Bezafibrate for 24 h showed significant increases in mRNA expression levels of fatty acid oxidation-related genes such as *Acs, Cpt1a,* and *Aox,* as shown in Fig. 1A. In addition, oxygen consumption rate (OCR) was significantly increased by Bezafibrate treatment (Fig. 1B). These findings indicate that Bezafibrate treatment increased fatty acid oxidation in Caco-2 cells. Under the same conditions, secretions of TG and apoB, which is a component of chylomicrons that transport food-derived lipids, into the basolateral side of Transwell® plates were decreased by Bezafibrate treatment (Fig. 1C, D). Bezafibrate did not increase TG accumulation in Caco-2 cells (data not shown). These findings suggest that enhancement of fatty acid oxidation by PPARα activation reduces TG secretion in Caco-2 cells.

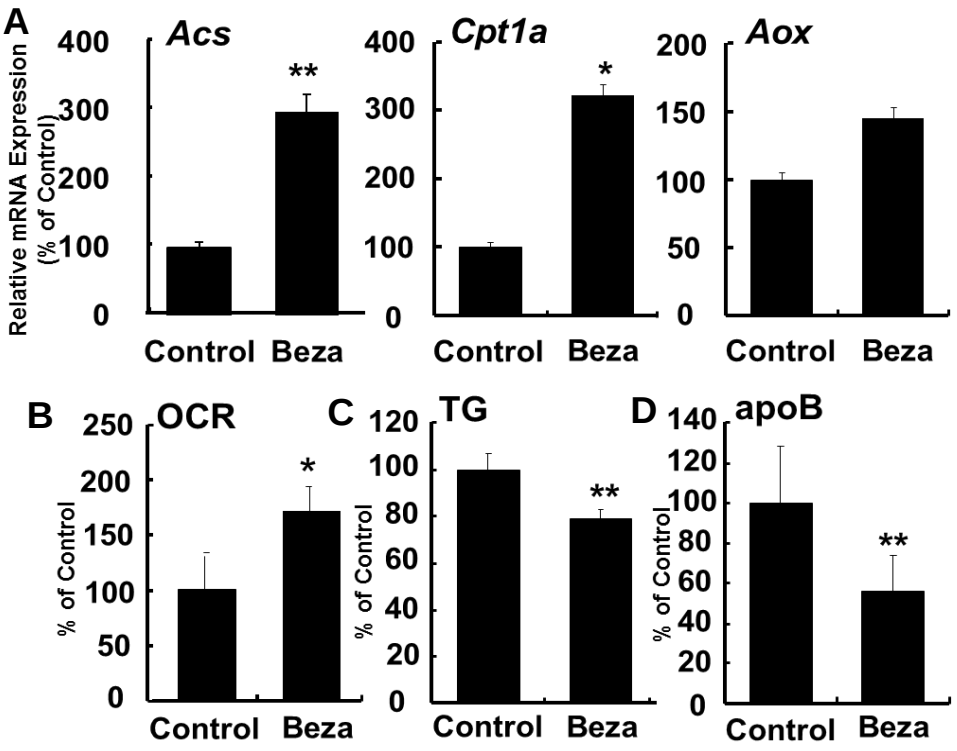


Fig. 1 Bezafibrate increases fatty acid oxidation and decreases TG and apoB secretion in Caco-2 cells

(A) mRNA expression levels of fatty acid oxidation-related genes (*Acs*, *Cpt1a*, and *Aox*) in Caco-2 cells treated with 50 μM Bezafibrate for 24 h. The copy number of each transcript was divided by that of *36B4* for normalization. (B) Oxygen consumption rate (OCR), (C) TG and (D) apoB secretion in Caco-2 cells treated with 50 μM Bezafibrate for 24 h, as described in the "MATERIALS AND METHODS" section. The value of a vehicle control was set at 100% and relative value is presented as fold induction compare with that of the vehicle control. The values are means ± S.E.M. of 6 samples. *P < 0.05 and **P < 0.01 compared with each vehicle control.

Bezafibrate enhanced fatty acid oxidation in intestinal epithelial cells of mice

To examine the *in vivo* effects of PPARα activation on intestinal fatty acid oxidation, C57BL/6 mice were fed HFD containing 0.2% Bezafibrate for 1 week. Bezafibrate increased in mRNA expression levels of fatty acid oxidation-related genes such as *Acs*, *Cpt1a*, and *Aox* in intestinal epithelial cells, as shown in Fig. 2A. Protein expression level of intestinal AOX was increased in mice fed HFD containing 0.2% Bezafibrate, as shown in Fig. 2B. Measurements of fatty acid oxidation using [^{14}C]-labeled palmitic acid revealed that productions of CO_2 and acid soluble metabolites (ASM), which are products of fatty acid oxidation including ketone bodies, were increased in intestinal epithelial cells of mice fed HFD containing 0.2% Bezafibrate (Fig. 2C, D). These findings indicate that Bezafibrate feeding for 1 week enhances fatty acid oxidation in intestinal epithelial cells of C57BL/6 mice.

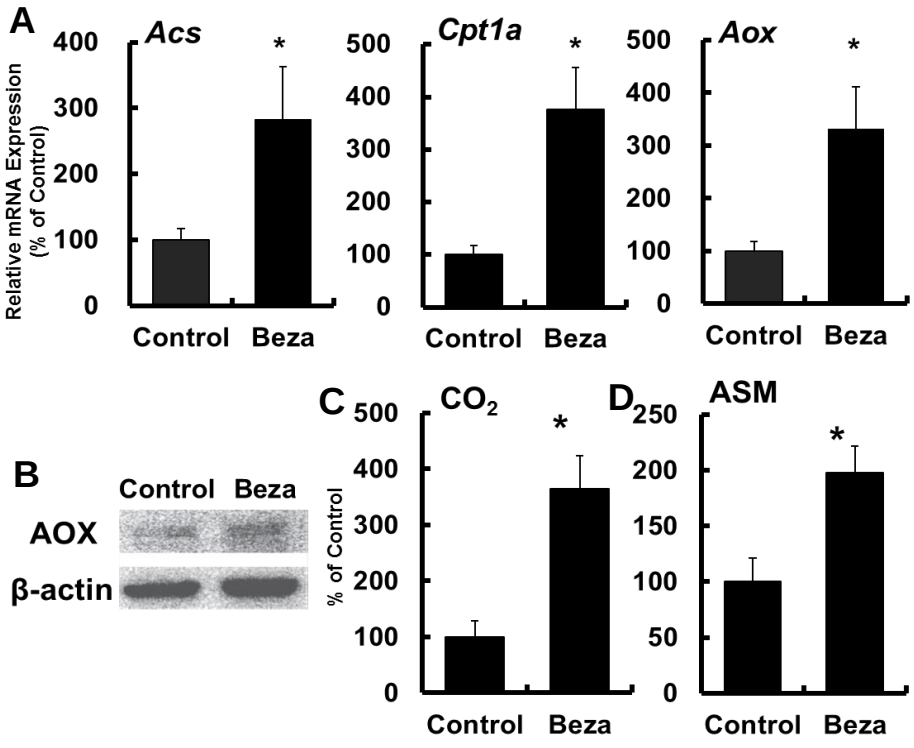


Fig. 2 Bezafibrate increases fatty acid oxidation and decreases TG and apoB secretion in C57BL/6 mice

(A) mRNA expression levels of fatty acid oxidation-related genes (*Acs*, *Cpt1a*, and *Aox*), (B) protein levels of Aox, and the production of CO_2 (C) and acid soluble metabolites (ASM) (D) in intestinal epithelial cells of C57BL/6J mice fed HFD containing 0.2% Bezafibrate for 1week, as described in the "MATERIALS AND METHODS" section. The value of a vehicle control was set at 100% and relative value is presented as fold induction compare with that of the vehicle control. The values are means ± S.E.M. of 8 mice. *P < 0.05 and **P < 0.01 compared with each vehicle control

Bezafibrate reduced TG secretion from intestinal epithelial cells and attenuated postprandial hyperlipidemia in mice

To study the effects of Bezafibrate-induced enhancement of fatty acid oxidation on postprandial hyperlipidemia, plasma TG concentration was measured after oral administration of olive oil (300 μL/mouse). After 16 h fasting to decrease basal TG concentration, olive oil was orally administered and plasma TG concentration was determined every 30 min after the administration. The plasma TG concentrations were lower in Bezafibrate-fed mice than in control mice throughout the experimental periods as shown in Fig. 3A. Initial peaks of plasma TG concentration 90 min after the administration were 198 and 91.9 mg/dl in the control and Bezafibrate-fed mice, respectively. Area under the curve (AUC) of plasma TG concentration in Bezafibrate-fed mice was smaller than that in control mice (Fig. 3B) The levels of plasma apoB48 were also decreased in Bezafibrate-fed mice (Fig. 3C). In addition, to investigate the effects of Bezafibrate on TG secretion from intestinal epithelial cells, plasma TG concentration was measured in mice that had been administered Tyloxapol, which is a TG clearance inhibitor, after oral administration of olive oil (300 μL/mouse). Plasma TG concentrations were decreased from 120 min after olive oil administration in mice fed HFD containing 0.2% Bezafibrate (Fig. 3D). The findings suggest that Bezafibrate-induced enhancement of fatty acid oxidation decreases TG secretion from intestinal epithelial cells, which attenuates postprandial hyperlipidemia.

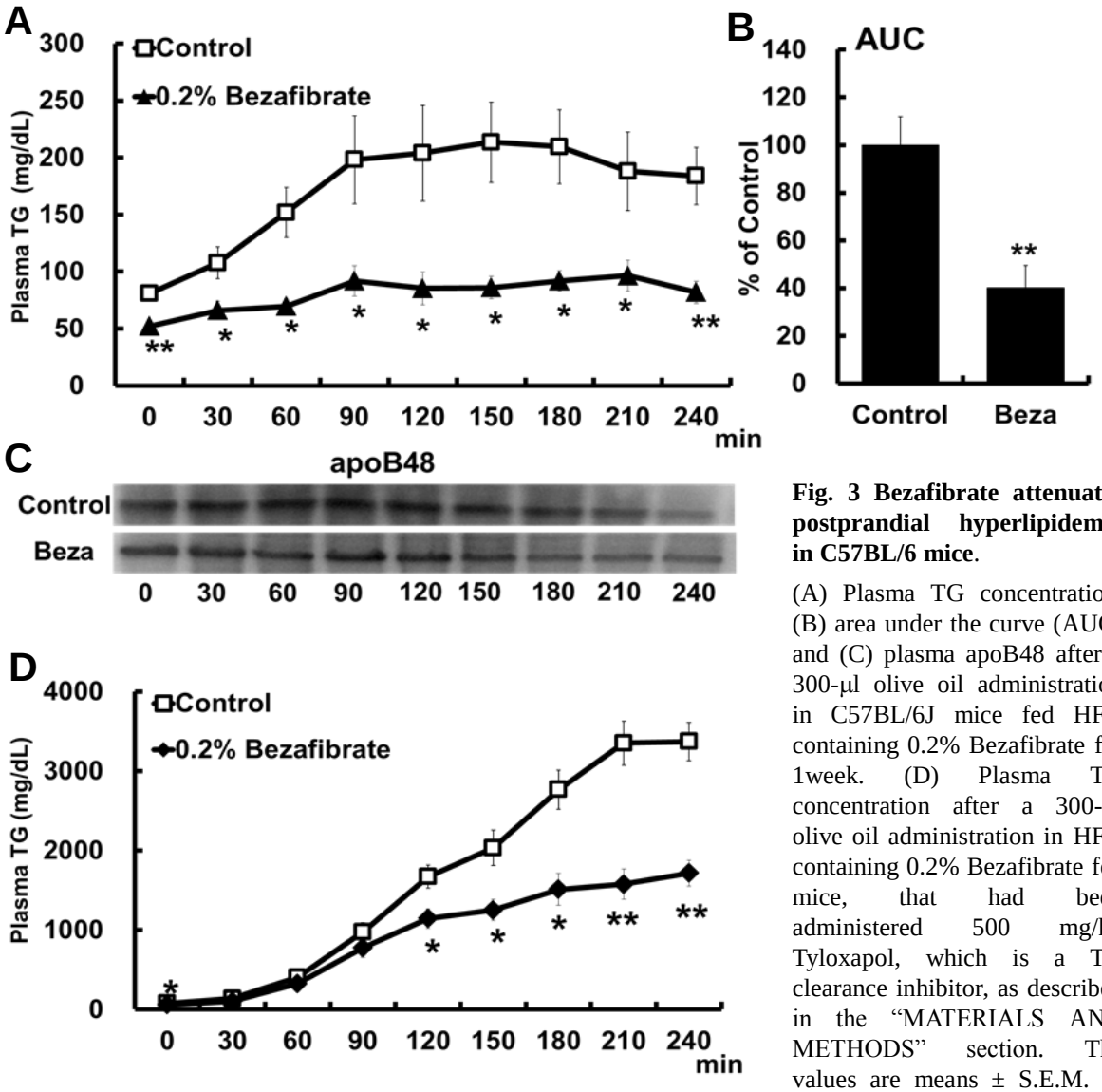


Fig. 3 Bezafibrate attenuates postprandial hyperlipidemia in C57BL/6 mice.

(A) Plasma TG concentration, (B) area under the curve (AUC) and (C) plasma apoB48 after a 300-μl olive oil administration in C57BL/6J mice fed HFD containing 0.2% Bezafibrate for 1week. (D) Plasma TG concentration after a 300-μl olive oil administration in HFD containing 0.2% Bezafibrate fed mice, that had been administered 500 mg/kg Tyloxapol, which is a TG clearance inhibitor, as described in the "MATERIALS AND METHODS" section. The values are means ± S.E.M. of 6-8 mice. *P < 0.05 and **P < 0.01 compared with each vehicle control.

Bezafibrate did not enhance intestinal fatty acid oxidation, reduce TG secretion from intestinal epithelial cells and attenuate postprandial hyperlipidemia in PPARα$^{-/-}$ mice

To elucidate whether the effects of Bezafibrate on intestinal fatty acid oxidation and postprandial hyperlipidemia in mice as above, were via the activation of PPARα, PPARα$^{-/-}$ mice were fed HFD containing 0.2% Bezafibrate for 1 week. Bezafibrate did not increase the mRNA expression levels of genes involved in fatty acid oxidation such as *Acs, Cpt1a,* and *Aox* in intestinal epithelial cells of PPARα$^{-/-}$ mice, as shown in Fig. 4A. Bezafibrate did not reduce plasma TG concentration in PPARα$^{-/-}$ mice after olive oil administration compared to control PPARα$^{-/-}$ mice (Fig. 4B). In addition, Bezafibrate did not decrease TG and apoB48 secretion from intestinal epithelial cells after olive oil administration in PPARα$^{-/-}$ mice that had been administered Tyloxapol (Fig. 4C, D). The findings suggest that enhancement of intestinal fatty acid oxidation by Bezafibrate decreases TG and apoB secretion from intestinal epithelial cells, which attenuates postprandial hyperlipidemia via PPARα activation in mice.

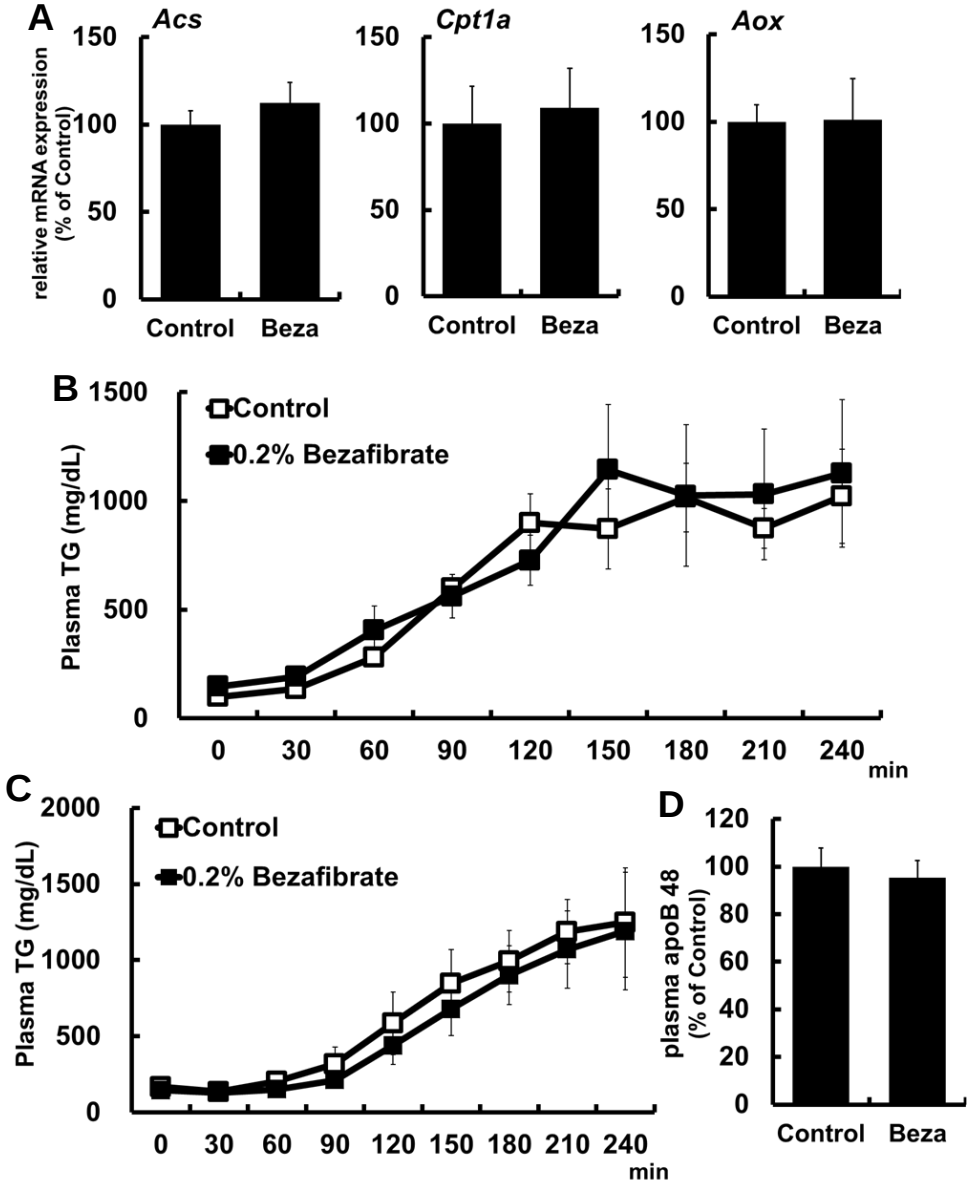


Fig. 4 Effects of Bezafibrate on postprandial lipid metabolism were abolished in PPARα$^{-/-}$ mice

(A) The mRNA expression levels of genes involved in fatty acid oxidation in intestinal epithelial cells of PPARα$^{-/-}$ mice fed HFD containing 0.2% Bezafibrate for 1week. (B) Plasma TG after a 300-μl olive oil administration in PPARα$^{-/-}$ mice fed HFD containing 0.2% Bezafibrate for 1week. (C) TG and (D) apoB48 secretion from intestinal epithelial cells after a 300-μl olive oil administration in HFD containing 0.2% Bezafibrate fed PPARα$^{-/-}$ mice, that had been injected Tyloxapol (0.5 g/kg body weight), as described in the "MATERIALS AND METHODS" section. The values are means ± S.E.M. of 6 mice.

DISCUSSION

Hyperlipidemia is considered to be a risk factor for cardiovascular diseases (10, 11). The liver mainly regulates circulating amounts of lipids under normal conditions through uptake and production of lipoproteins. However, under postprandial conditions, lipid absorption into and transport from intestinal epithelial tissue are important for regulating serum lipid levels. Postprandial serum lipid levels have been shown to have a stronger positive correlation with coronary artery disease than the fasting serum lipid levels (11). Therefore, reduction of postprandial serum lipid levels is effective for prevention of cardiovascular diseases. For prevention of absorption of dietary lipids, pancreatic lipase inhibitors have been used (18, 19). Orlistat, a pancreatic lipase inhibitor used for the treatment of obesity, is an effective drug reducing lipid absorption via intestinal epithelial cells and preventing weight gain in the treatment of obesity in the primary care setting. However, such inhibition of pancreatic lipase causes fecal urgency, oily spotting, and fatty/oily stool (20). Our present study indicates that enhancement of fatty acid oxidation in intestinal epithelial cells attenuates postprandial hyperlipidemia via PPARα activation in mice. There has been no report showing such fecal side effects in the case of PPARα agonist administration. Therefore, the present study suggests a possibility that enhancement of fatty acid oxidation by PPARα activation is a novel target for prevention of cardiovascular diseases.

We have demonstrated that many food-derived compounds function as PPARα agonists with anti-diabetic and anti-hyperlipidemic effects in various tissues such as the liver and skeletal muscle (5-8, 21). Therefore, food-derived compounds with PPARα activity could enhance fatty acid oxidation in intestinal epithelial cells, which could reduce postprandial hyperlipidemia and lipid accumulation in the liver and skeletal

muscle in mice, although further investigations are needed to elucidate the contribution. Natural compounds activating PPARα generally show lower activities than synthetic agonists such as Bezafibrate (22). This is because natural compounds are transformed into metabolites that have much lower effects in the liver before entering the whole-body circulation. In this sense, the intestinal effects of natural compounds before being metabolized in the liver are very significant when considering the functions of food-derived compounds. The present study could shed light on importance of intestinal lipid metabolism as a primary target of PPARα agonists.

REFERENCES

1. P. Lefebvre, G. Chinetti, J.C. Fruchart, B. Staels. 2006. Sorting out the roles of PPAR-alpha in energy metabolism and vascular homeostasis. *J. Clin. Invest.* 116: 571–580.
2. N. Takahashi, T. Goto, S. Hirai, T. Uemura, T. Kawada. 2009. Genome science of lipid metabolism and obesity. *Forum Nutri.* 61:25–38.
3. S. Mandard, M. Müllar, S. Kersten. 2004. Peroxisome proliferator-activated receptors-alpha target genes. *Cell. Mol. Life Sci.* 61:393–416.
4. K. Schoonjans, B. Staels, J. Auwerx. 1996. Role of the peroxisome proliferatoractivated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *J. Lipid Res.* 37: 907–925.
5. Kang MS, Hirai S, Goto T, Kuroyanagi K, Lee JY, Uemura T, Ezaki Y, Takahashi N, Kawada T. 2008. Dehydroabietic acid, a phytochemical, acts as ligand for PPARs in macrophages and adipocytes to regulate inflammation. *Biochem Biophys Res*

Commun. 369:333-8.

6. T. Goto, N. Takahashi, S. Kato, K. Egawa, S. Ebisu, T. Moriyama, T. Fushiki, T. Kawada. 2005. Phytol directly activates PPAR-alpha and regulates gene expression involved in lipid metabolism in PPAR-alpha-expressing HepG2 hepatocytes. *Biochem. Biophys. Res. Commun*. 337:440–445.
7. N. Takahashi, K.M. Kang, K. Kuroyanagi, T. Goto, S. Hirai, K. Ohyama, J.Y. Lee, R.Yu, M. Yano, T. Sasaki, S. Murakami, T. Kawada. 2008. Auraptene, a citrus fruit compound, regulates gene expression as a PPAR-alpha agonist in HepG2 hepatocytes. *BioFactors* .33:25–32.
8. M.S. Kang, S. Hirai, T. Goto, K. Kuroyanagi, I.Y. Kim, K. Ohyama, T. Uemura, J.Y. Lee, T. Sakamoto, Y. Ezaki, R. Yu, N. Takahashi, T. Kawada. 2009. Dehydroabietic acid a phytochemical acts as ligand for PPARs in macrophages and adipocytes to regulate inflammation of mouse adipose tissues. *BioFactors* . 35:442–448.
9. T. Goto, J.Y. Lee, A. Teraminami, Y.I. Kim, S. Hirai, T. Uemura, H. Inoue, N. Takahashi, T. Kawada. 2011. Activation of PPAR-alpha stimulates both differentiation and fatty acid oxidation in adipocytes. *J. Lipid Res.* 52:873–884.
10. S. Yamashita, K. Hirano, T. Kuwasako, M. Janabi, Y. Toyama, M. Ishigami, N. Sakai. 2007. Physiological and pathological roles of a multi-ligand receptor CD36 in atherogenesis; insights from CD36-deficient patients. *Mol. Cell. Biochem.* 299:19–22.
11. J.R. Patsch, G. Miesenbock, T. Hopferwieser, V. Muhlberger, E. Knapp, J.K. Dunn, A.M. Gotto Jr., W. Patsch. 1992. Relation of triglyceride metabolism and coronary artery disease. *Arterioscl. Thronb.* 12 :1336–1345.
12. Smith D, Watts GF, Dane-Stewart C, Mamo JC. 1999. Post-prandial chylomicron

response may be predicted by a single measurement of plasma apolipoprotein B48 in the fasting state. *Eur J Clin Investig.* 29:204–9.

13. Chan DC, Pang J, Romic G, Watts GF. 2013. Postprandial hypertriglyceridemia and cardiovascular disease: current and future therapies. *Curr Atheroscler Rep.* 15:309.
14. Uemura T, Hirai S, Mizoguchi N, Goto T, Lee JY, Taketani K, Nakano Y, Shono J, Hoshino S, Tsuge N, Narukami T, Takahashi N, Kawada T. 2010. Diosgenin present in fenugreek improves glucose metabolism by promoting adipocyte differentiation and inhibiting inflammation in adipose tissues. *Mol Nutr Food Res*. 54:1596-608.
15. Kim YI, Hirai S, Goto T, Ohyane C, Takahashi H, Tsugane T, Konishi C, Fujii T, Inai S, Iijima Y, Aoki K, Shibata D, Takahashi N, Kawada T. 2012. Potent PPARα activator derived from tomato juice, 13-oxo-9,11-octadecadienoic acid, decreases plasma and hepatic triglyceride in obese diabetic mice. PLoS One. 7:e31317.
16. M. Watanabe, S.M. Houten, C. Mataki, M.A. Christoffolete, B.W. Kim, H. Sato, N. Messaddeq, J.W. Harney, O. Ezaki, T. Kodama, K. Schoonjans, A.C. Bianco, J. Auwerx. 2006. Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature.* 439: 484–489.
17. Y. Mochizuki, M. Maebuchi, M. Kohno, M. Hirotsuka, H. Wadahama, T. Moriyama, T. Kawada, R. Urade. 2009. Changes in lipid metabolism by soy betaconglycinin-derived peptides in HepG2 cells. *J. Agric. Food. Chem.* 57: 1473–1480.
18. A. Ballinger, S.R. Peikin. 2002. Orlistat: its current status as an anti-obesity drug. *Eur. J. Pharmacol.* 440: 109–117.
19. K.H. Lucas, B. Kaplan-Machlis. 2001. Orlistat: a novel weight loss therapy. *Ann. Pharmacother.* 35: 314–328.
20. J. Hauptman, C. Lucas, M.N. Boldrin, H. Collins, K.R. Segal. 2000. Orlistat in the

long term treatment of obesity in primary care settings. *Arch. Fam. Med.* 9:160–167.

21. K. Kuroyanagi, M.S. Kang, T. Goto, S. Hirai, K. Ohyama, T. Kusudo, R. Yu, M. Yano, T. Sasaki, N. Takahashi, T. Kawada. 2008. Citrus auraptene acts as an agonist for PPARs and enhances adiponectin production and MCP-1 reduction in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* 366: 219–225.
22. T. Goto, N. Takahashi, S. Hirai, T. Kawada. 2010. Various terpenoids derived from herbal and dietary plants function as PPAR modulators and regulate carbohydrate and lipid metabolism. *PPAR Res*. Article ID 483958.

This research was originally published by Elsevier. The definitive version has been published in Biochemical and Biophysical Research Communications, Volume 410, Issue 1, Pages 1–6, 24 June 2011, doi: 10.1016/j.bbrc.2011.05.057.

Chapter 2

Studies on effects of PPARα activation on intestinal fatty acid oxidation and postprandial hyperlipidemia in obesity

INTRODUCTION

Several changes in human nutrition have occurred these last decades in most parts of the world, among which a significant increase in caloric intake and an increase in saturated fatty acid intake (1). These changes occurred simultaneously with a rise in metabolic diseases, such as obesity, metabolic syndrome, and diabetes, that are risk factors of atherosclerosis and cardiovascular diseases. Several studies have established that lipid metabolism is impaired in metabolic diseases. For example, hepatic fatty acid synthesis is enhanced under diabetic conditions, so that triglyceride (TG) accumulates in hepatocytes leading to the development of fatty liver, increasing the risk of cardiovascular diseases (2, 3). Therefore, it is crucial for preventing cardiovascular diseases associated with diabetes and obesity, to improve dyslipidemia, particularly to decrease fasting serum TG level, which has been considered to be a risk of cardiovascular diseases (4). However, it has recently been demonstrated that postprandial TG level is associated with the risk more than fasting serum TG level (5). Therefore, regulation of postprandial hyperlipidemia is thought to be important for prevention of cardiovascular diseases.

Peroxisome proliferator-activated receptor (PPAR)-α is a member of the nuclear receptor superfamily, which is activated by small hydrophobic compounds such as fatty

acid and its derivatives (6). PPARα is expressed in peripheral tissues with a high potential of lipid metabolism including the liver and skeletal muscle, whose activation enhances fatty acid oxidation and decreases circulating TG level (7, 8). Hence, synthetic PPARα agonists such as fibrates are used for hypolipidemic drug. Interestingly, our recent work has demonstrated that PPARα is expressed in white adipose tissues (WATs), which have been thought to be a lipid storage organ, and enhances fatty acid oxidation to regulate energy homeostasis (9). Therefore, PPARα is thought to be very effective for managing obese conditions. However, obesity reduces PPARα expression level in WATs and the small intestine, so that the anti-obese effect of PPARα is reduced under obese conditions (9, 10). Although the molecular mechanism of the reduction of PPARα expression in obesity remains unknown, it is possible that even decreased PPARα activities improves dyslipidemia in obese conditions.

The small intestine is exposed to amount of orally ingested substances in postprandial state. Amounts of TG and fatty acid absorbed in our bodies affect the development of cardiovascular diseases (11, 12). After the absorption of lipid, chylomicrons assembled together with resynthesized TG and apolipoprotein B in intestinal epithelial cells, are transported into circulation via lymph vessels (13). Therefore, regulation of intestinal lipid metabolism could have a great impact on plasma TG level via control of chylomicron production. We and others have shown that PPARα activation in intestinal epithelial cells attenuates postprandial hyperlipidemia by enhancing fatty acid oxidation (14, 15). However, it is unclear whether the effects of intestinal PPARα activation on postprandial hyperlipidemia are observed under obese conditions. In the present study, we elucidated effects of intestinal PPARα activation on postprandial hyperlipidemia in KK-Ay mice, which are obese diabetic model mice. Dietary fat is known to be absorbed

in the jejunum, in well-differentiated intestinal epithelial cells located in the upper 2/3 of the villi under normal condition (16). A high-fat diet/meal may trigger an overflow of dietary fat absorption capacity in proximal part of intestine and thus trigger the recruitment of distal part of the intestine for their absorption (17, 18). In addition, *Pparα* are highly expressed in a wide range of small intestine (19). Therefore, it is unknown where *Pparα* could affect postprandial hyperlipidemia in the small intestine. Therefore, we examined the contribution of *Pparα* in proximal and distal intestine to postprandial lipid metabolism.

MATERIALS AND METHODS

Animal and diet

Male KK-Ay mice, a useful model of obesity and diabetes (20), were purchased from CLEA Japan (Tokyo, Japan). The mice at 7 weeks of age were bred for a week under high-fat diet (HFD) consisting of 60% (kcal%) fat -fed conditions for habituation. Their plasma glucose level was 557.3 ± 18.2 mg/dl, indicating diabetic conditions. The mice were kept in individual cages in a temperature-controlled room at 24 ± 1^{o}C and maintained under a constant 12-h light/dark cycle. All the animal experiments were approved by Kyoto University Animal Care Committee (approval ID: No. 22-53). The KK-Ay mice (8 weeks old) were divided into two groups by average body weight and serum TG level after the habituation. Each group was maintained on 60% HFD or HFD containing 0.2% (w/w) Bezafibrate for a week. The energy intake of all the mice was adjusted by pair feeding. Thus, the food intake levels and body weights of each group were almost the same (Fig. 1).

Isolation of intestinal epithelial cells

For the measurement of gene expression, intestinal epithelial tissue was collected with a slide glass after washing twice with cold PBS. For the measurement of fatty acid oxidation, intestinal epithelial cells were isolated from the proximal 1/2 (upper) and distal 1/2 (lower) of the intestine and incubated in 1 mg/ml collagenase IA/HBSS for 40 min. They were washed with 1% FBS/DMEM three times and used for the experiments.

Gene expression

Total RNA samples were prepared from collected intestinal epithelial cells using an SV total RNA isolation system (Promega, WI, USA) in accordance with the manufacturer's protocol. To quantify mRNA expression, PCR was performed using a fluorescence temperature cycler (LightCycler System: Roche Diagnostics, Mannheim, Germany), as previously described (21, 22). Primer sets were designed using a PCR primer selection program at the web site of the Virtual Genomic Center from the GenBank database, and their sequences are shown in Table 1. To compare mRNA expression levels among samples, the copy number of each transcript was divided by that of *36B4* showing a constant expression level. All mRNA expression levels are presented as the percentage relative to the control in each experiment, as previously described (14).

Table 1 Oligonucleotide primers used for RNA analysis

Gene	Forward primer	Reverse primer
mouse *36B4*	CTTTGGCGGGATTAGTCGAAG	TGTGTGTCTGCAGATCGGGTAC
mouse *Acs*	GAGATATTCTGGCCACCGATCA	ACGTATCCCTGGACTAGGACCG
mouse *Cpt1a*	GGCCTCTGTGGTACACGACAA	CTCAGTGGGAGCGACTCTTCA
mouse *Aox*	CAGGATCCGACTGTTTACC	CTTGTTCGCGCAAGTGAGG
mouse *Pparα*	TCAGGGTACCACTACGGAGT	CTTGGCATTCTTCCAAAGCG
mouse *Cd36*	GATGTGGAACCCATAACTGGATTCAC	GGTCCCAGTCTCATTTAGCCACAGT

Fatty acid oxidation in intestinal epithelial cells of KK-Ay mice

The intestinal epithelial cells isolated from HFD fed control mice or HFD containing Bezafibrate-fed mice were incubated in DMEM containing 200 μM palmitic acid, 0.01% fatty acid-free BSA, 200 μM 1-carnitine, and [^{14}C]-palmitic acid (37 kBq) (American Radiolabeled Chemicals, MO, USA) for 2 h. Fatty acid oxidation products were assessed as previously described (14). Briefly, the labeling medium containing intestinal epithelial cells was transferred to a 50-ml polypropylene tube. An uncapped 2-ml sample tube containing a piece of filter paper soaked in 3 N NaOH was placed inside a 50-ml sample tube. After the tube was sealed, 200 μl of 12 N HCl was added to the medium sample to release [^{14}C]-CO_2. The tube was then incubated at 37°C for 24 h. The saturated filter paper containing trapped [^{14}C]-CO_2 was assessed for radioactivity in a liquid scintillation counter (LS6500, Beckman Coulter, CA, USA). The acidified medium was centrifuged and 200 μl of supernatant was assessed for the amount of [^{14}C]-labeled acid soluble metabolites, which include labeled ketone bodies. Protein concentration was determined using a protein assay kit (Bio-Rad, CA, USA).

Postprandial triglyceridemic response

Plasma TG concentration was measured after the oral administration of olive oil as previously described (14). Briefly, after 16 h of fasting to decrease basal TG concentration, olive oil (300 μl/mouse) was orally administered and plasma was collected from the tail vein of non-anesthetized mice every 30 min up to 240 min after the administration. For the determination of plasma TG concentration, we used triglyceride E Test WAKO (Wako, Osaka, Japan)

Statistical analysis

The data were presented as means ± S.E.M. and statistically analyzed using one-way ANOVA when their variances were heterogeneous and unpaired t-test. Differences were considered significant at $P < 0.05$.

RESULTS

Bezafibrate increased fatty acid oxidation in proximal intestine of KK-Ay mice

To investigate effects of Bezafibrate on intestinal lipid metabolism under obese diabetic conditions, we used 9-week-old KK-Ay mice fed HFD for 1 week. The food intake levels and body weights were almost the same in both groups (Fig. 1A, B). The mRNA expression levels of genes involved in fatty acid oxidation such as *Acs*, *Cpt1a*, *Aox*, and *Cd36* were higher in proximal intestine of the Bezafibrate fed mice than those of HFD-fed control mice (Fig. 2A-D). In addition, Bezafibrate induced mRNA expression of *Pparα* in proximal intestinal epithelial cells as shown Fig. 2E. Productions of CO_2 and acid soluble metabolites (ASM) including ketone bodies, were also higher in Bezafibrate-fed mice than in control mice (Fig. 2F and G), suggesting that fatty acid oxidation was enhanced in proximal intestinal epithelial cells of Bezafibrate-fed mice. Meanwhile, the expression levels of lipogenic genes, such as *Srebp-1c* and *Dgat*, showed slightly increase in proximal intestinal epithelial cells of Bezafibrate-fed mice compared to those of control mice, although the difference did not reach statistical significance (data not shown). These results indicate that Bezafibrate enhances fatty acid oxidation in proximal intestinal epithelial cells under obese diabetic conditions.

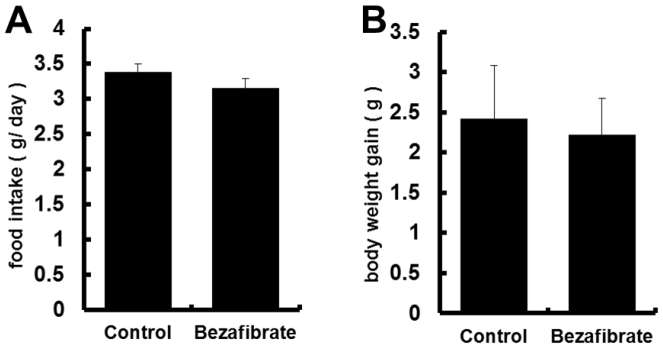


Fig. 1 food intake and body weight in KK-Ay mice
(A) average food intake, and (B) body weight gain in HFD-fed control mice and HFD containing 0.2% Bezafibrate-fed KK-Ay mice
The values are means ± S.E.M. of 6 mice.

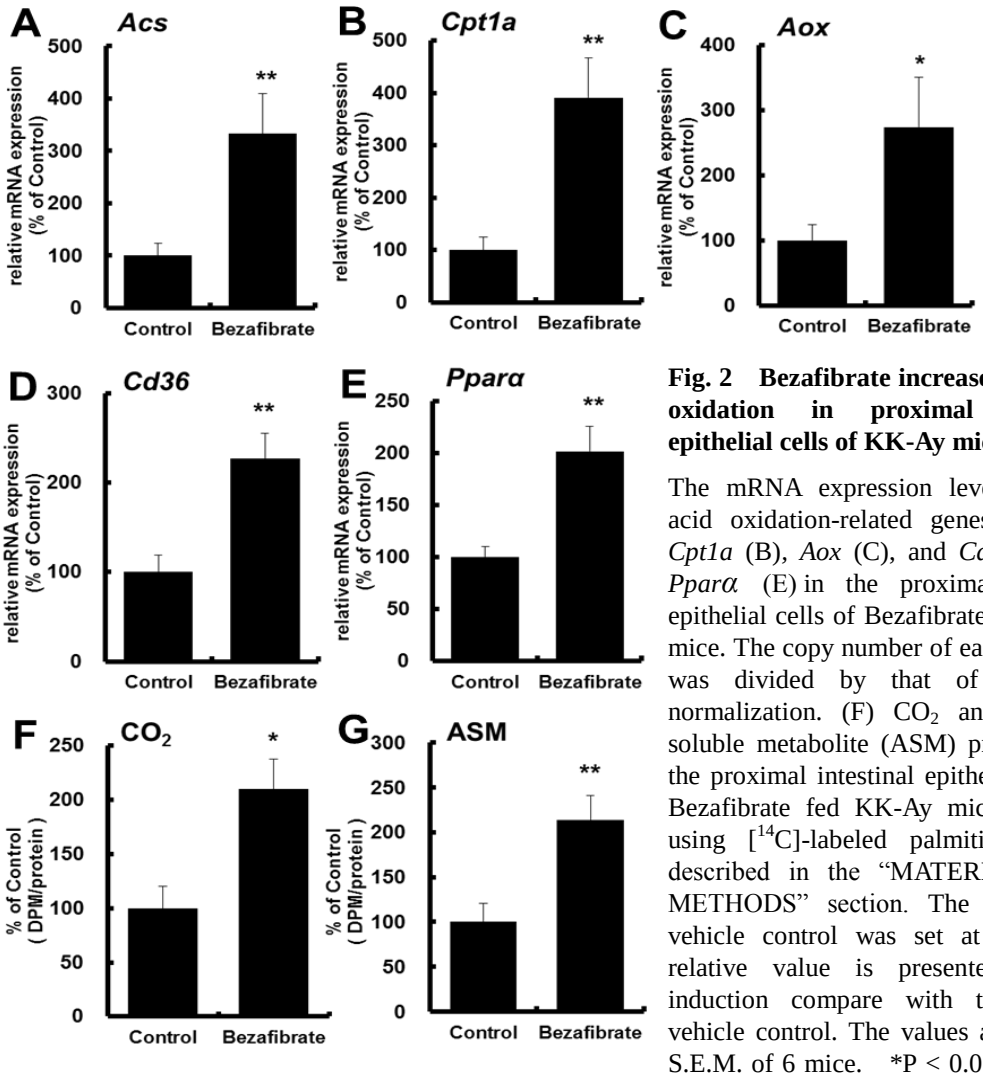


Fig. 2 Bezafibrate increases fatty acid oxidation in proximal intestinal epithelial cells of KK-Ay mice

The mRNA expression levels of fatty acid oxidation-related genes, *Acs* (A), *Cpt1a* (B), *Aox* (C), and *Cd36* (D) and *Pparα* (E) in the proximal intestinal epithelial cells of Bezafibrate fed KK-Ay mice. The copy number of each transcript was divided by that of *36B4* for normalization. (F) CO_2 and (G) acid soluble metabolite (ASM) production in the proximal intestinal epithelial cells of Bezafibrate fed KK-Ay mice measured using [^{14}C]-labeled palmitic acid, as described in the "MATERIALS AND METHODS" section. The value of a vehicle control was set at 100% and relative value is presented as fold induction compare with that of the vehicle control. The values are means ± S.E.M. of 6 mice. *P < 0.05 and **P < 0.01 compared with each vehicle control.

Bezafibrate did not increase fatty acid oxidation in distal intestine of KK-Ay mice

We have examined only proximal intestine in measurements of gene expression and fatty acid oxidation (14). However, lipid absorption occurs in not only proximal intestine but also distal intestine (17). Therefore, we measured the gene expression levels and fatty acid oxidation in distal intestinal epithelial cells. As shown in Fig. 3A-D, the expression levels of target genes of *Pparα* and *Pparα* were not increased by Bezafibrate, although the expression levels of *Pparα* were similar in both proximal and distal intestinal epithelial cells of control mice (data not shown). The mRNA expression levels of *Cd36* were statistically significant induced in distal intestinal epithelial cells (Fig. 3E), however, expression levels of *Cd36* were lower in distal intestine than in proximal ones of control mice. Moreover, the effect of Bezafibrate on induction of *Cd36* was smaller in distal intestine than in proximal intestine. There was no significant difference in production of CO_2 and ASM between distal intestine of Bezafibrate-fed mice and that of control mice (Fig. 3F and G). The expression levels of lipogenic genes, such as *Srebp-1c* and *Dgat*, were similar between both groups (data not shown). These findings suggest that Bezafibrate does not induce fatty acid oxidation in distal intestinal epithelial cells under obese diabetic conditions.

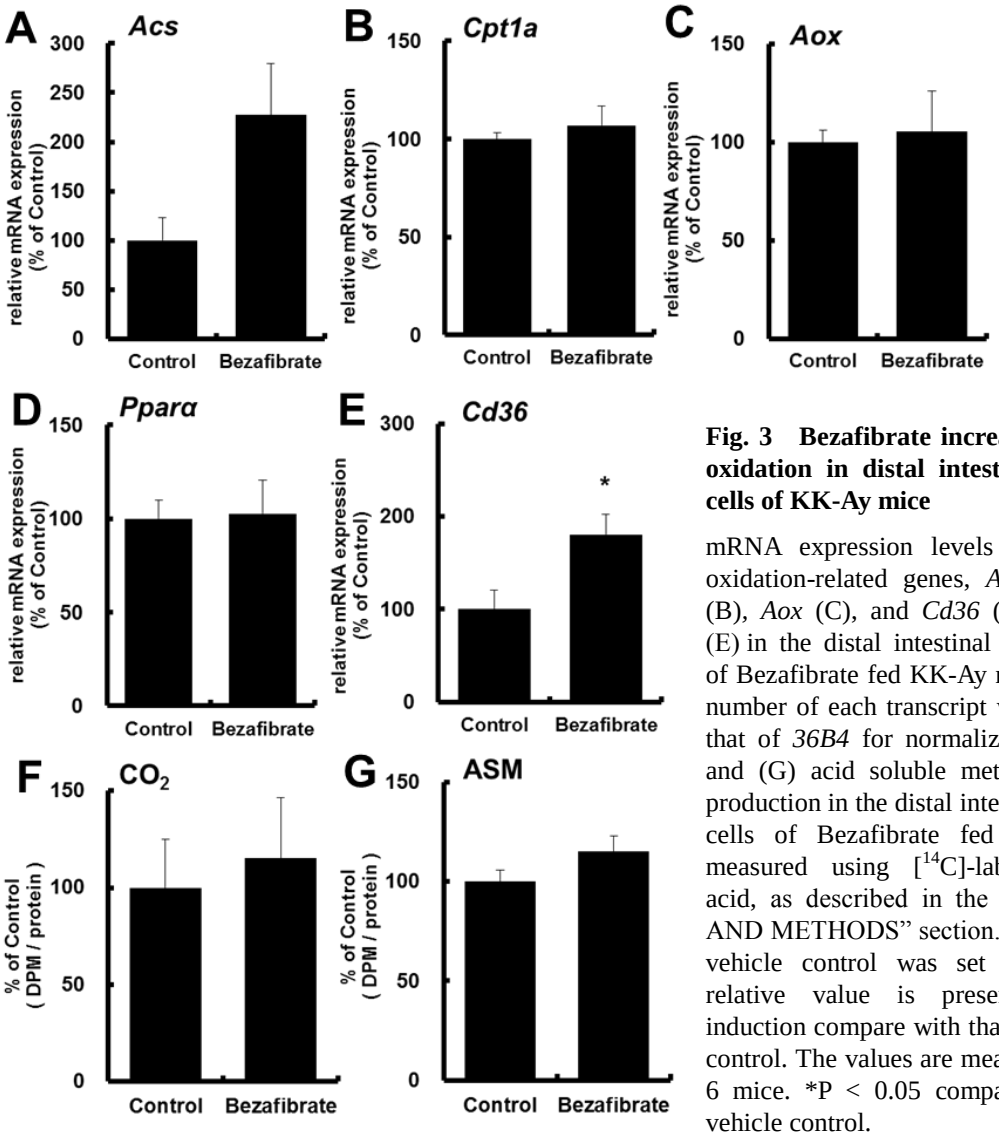


Fig. 3 Bezafibrate increases fatty acid oxidation in distal intestinal epithelial cells of KK-Ay mice

mRNA expression levels of fatty acid oxidation-related genes, *Acs* (A), *Cpt1a* (B), *Aox* (C), and *Cd36* (D) and *Pparα* (E) in the distal intestinal epithelial cells of Bezafibrate fed KK-Ay mice. The copy number of each transcript was divided by that of *36B4* for normalization. (F) CO_2 and (G) acid soluble metabolite (ASM) production in the distal intestinal epithelial cells of Bezafibrate fed KK-Ay mice measured using [^{14}C]-labeled palmitic acid, as described in the "MATERIALS AND METHODS" section. The value of a vehicle control was set at 100% and relative value is presented as fold induction compare with that of the vehicle control. The values are means ± S.E.M. of 6 mice. *P < 0.05 compared with each vehicle control.

PPARα activation in intestinal epithelial cells reduced postprandial hyperlipidemia in KK-Ay mice

To examine effects of Bezafibrate on postprandial triglyceridemic response under obese diabetic conditions, plasma TG concentration was measured after oral administration of olive oil (300 μL/mouse) in KK-Ay mice. At 0 min after 16h fasting, there was no difference in plasma TG levels between both groups. In control mice, plasma TG levels peaked 120-150 min after the administration (Fig. 4A). In contrast, plasma TG levels were significantly decreased in Bezafibrate-fed mice from 90 min up to 240 min after olive oil administration. The area under the curve (AUC) was also smaller in Bezafibrate-fed mice than in control mice (Fig. 4B). These findings indicate that Bezafibrate reduces postprandial hyperlipidemia in KK-Ay mice. These results suggest that enhancement of fatty acid oxidation in proximal intestinal epithelial cells contributes to attenuating postprandial hyperlipidemia under obese diabetic conditions.

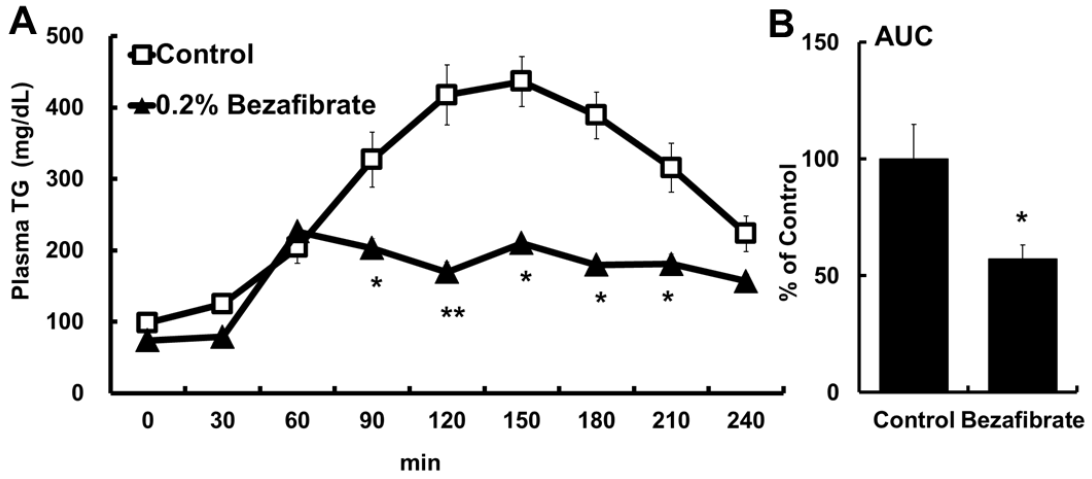


Fig. 4 Bezafibrate attenuates postprandial hyperlipidemia in KK-Ay mice

(A) Postprandial triglyceridemic response was measured in KK-Ay mice fed HFD containing 0.2% Bezafibrate for 1week. Plasma TG concentration at 0 min, every 30 min to 240 min after a 300-μl olive oil administration in KK-Ay mice. (B) Area under the curve (AUC) of postprandial triglyceridemic response, as described in the "MATERIALS AND METHODS" section. The values are means ± S.E.M. of 6 mice. *P < 0.05 and **P < 0.01 compared with each vehicle control.

DISCUSSION

It is well known that obesity and diabetes change lipid metabolism in various tissues including the liver and small intestine (10, 23). For example, fatty acid synthesis is enhanced under obese diabetic conditions in both liver (2) and intestine (10, 23). In addition, obesity reduces PPARα expression level in WATs and the small intestine, so that the anti-obese effect of PPARα is reduced under obese conditions (9, 10). Previously, we have elucidated that PPARα activation attenuates postprandial hyperlipidemia through the increase in the intestinal fatty acid oxidation of lean C57BL /6 mice (14). However, it remained unknown whether the same effects of PPARα activation on postprandial hyperlipidemia could be observed under obese diabetic conditions. The present study showed that PPARα activation enhanced fatty acid oxidation in proximal intestinal epithelial cells and attenuated postprandial hyperlipidemia in obese diabetic KK-Ay mice. However, the gene expression involved in lipogenesis, such as *Srebp-1c* and *Dgat*, were increased in proximal intestine. These results suggest that the enhancement of fatty acid oxidation exceeds induction of lipogenesis in proximal intestine of KK-Ay mice by Bezafibrate. Recently, postprandial hyperlipidemia has been thought to be a risk factor for atherosclerosis rather than fasting blood TG level (5). In obese diabetic conditions, such a risk is seemed to increase because the peak plasma TG level after the olive oil administration was higher in the KK-Ay mice than in the C57BL mice (402 and 209 mg/dl in the KK-Ay mice, as shown in Fig. 4, and C57BL mice, respectively (14)). Therefore, attenuation of postprandial hyperlipidemia via PPARα activation could be an effective strategy for prevention and improvement of atherosclerosis even in obesity.

Lipid absorption occurs in not only proximal intestine but also distal intestine, especially after a fatty meal intake (17). The expression levels of transporter proteins for fatty acid and cholesterol such as CD36 and ABC transporter family proteins differ between proximal and distal intestine (24, 25), suggesting that the amounts of absorbed fatty acid are also different between them. In our previous report, we have examined the effects of PPARα activation in only proximal intestinal epithelial cells (14), and the contribution of PPARα in distal intestine to postprandial lipid metabolism remained unknown. Therefore, we compared the effects of PPARα activation between proximal and distal intestine in the present study (Fig. 2, 3). We demonstrated here that Bezafibrate increased mRNA expression of genes involved in fatty acid oxidation in proximal intestine, but not in distal intestine. However, there was no difference in mRNA expression of *Pparα* between proximal and distal intestine of control mice in this experiment. These findings suggest that the induction of gene expression is regulated in a site-specific manner in the small intestine, and PPARα activation in proximal intestine is sufficient to reduce postprandial hyperlipidemia. This suggestion is very important for the development of drug targeting intestinal lipid metabolism.

Circulating lipid amounts are affected by lipid absorbed through the small intestine and clearance into other peripheral tissues, such as the liver. Many reports have demonstrated that the increase in hepatic fatty acid oxidation induced by PPARα activation results in decrease in circulating lipid amounts (26, 27). However, especially early in postprandial state, increase in plasma TG levels comes from amount of dietary fat absorbed in the small intestine, and contribution by the liver to circulating lipid levels is thought to be small (28, 29). Therefore, intestinal lipid metabolism would be

more important for the circulating lipid levels than the hepatic lipid metabolism under our experimental condition.

In conclusion, PPARα activation in intestinal epithelial cells attenuates postprandial hyperlipidemia even under obese diabetic conditions. The effects of PPARα are mediated by proximal intestinal epithelial cells. Because the small intestine is exposed to orally ingested substances in postprandial state and the surface area of the villus mucosa is quite large, the regulation of intestinal postprandial lipid metabolism by chemicals or food ingredients could become efficient for preventing the development of cardiovascular disease even in obesity.

REFERENCES

1. Margetts B. 2003. Feedback on WHO/FAO global report on diet, nutrition, and noncommunicable diseases. *Public Health Nutr.* 6: 423–429.
2. Shimomura I, Bashmakov Y, Horton JD. 1999. Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. *J Biol Chem.* 274:30028-32.
3. Memon RA, Grunfeld C, Moser AH, Feingold KR. 1994. Fatty acid synthesis in obese insulin resistant diabetic mice. *Horm. Metab Res.* 26:85–7.
4. Yamashita S, Hirano K, Kuwasako T, Janabi M, Toyama Y, Ishigami M, Sakai N. 2007. Physiological and pathological roles of a multi-ligand receptor CD36 in atherogenesis; insights from CD36-deficient patients. *Mol Cell Biochem.* 299:19-22.
5. Patsch JR, Miesenbock G, Hopferwieser T, Muhlberger V, Knapp E, Dunn JK, Gotto AM Jr, Patsch W. 1992. Relation of triglyceride metabolism and coronary artery disease. *Arterioscl Thromb.* 12:1336-45.

6. Lefebvre P, Chinetti G, Fruchart JC, Staels B. 2006. Sorting out the roles of PPARα in energy metabolism and vascular homeostasis. *J Clin Invest.* 116:571-80.
7. Mandard S, Müllar M, Kersten S. 2004. Peroxisome proliferator-activated receptor-alpha target genes. *Cell Mol Life Sci.* 61:393-416.
8. Schoonjans K, Staels B, Auwerx J. 1996. Role of PPAR in mediating the effects of fibrates and fatty acids on gene expression. *J Lipid Res*. 37:907-25.
9. Goto T, Teraminami A, Lee JY, Hirai S, Uemura T, Kim YI, Takahashi N, Kawada T. 2011. Activation of PPARα stimulates both differentiation and fatty acid oxidation in adipocytes. *J Lipid Res.* 52: 873-84.
10. Kondo H, Minegishi Y, Komine Y, Mori T, Matsumoto I, Abe K, Tokimitsu I, Hase T, Murase T. 2006. Differential regulation of intestinal lipid metabolism-related genes in obesity-resistant A/J vs. obesity-prone C57BL/6J mice. *Am J Physiol Endocrinol Metab.* 291:E1092-9.
11. Stanley WC, Dabkowski ER, Ribeiro RF Jr, O'Connell KA. 2012. Dietary fat and heart failure: moving from lipotoxicity to lipoprotection. *Circ Res.* 110:764-76.
12. Cascio G, Schiera G, Di Liegro I. 2012. Dietary fatty acids in metabolic syndrome, diabetes and cardiovascular diseases. *Curr Diabetes Rev.* 8:2-17.
13. Chan DC, Pang J, Romic G, Watts GF. 2013. Postprandial hypertriglyceridemia and cardiovascular disease: current and future therapies. *Curr Atheroscler Rep.* 15:309.
14. Kimura R, Takahashi N, Murota K, Yamada Y, Kanzaki N, Murakami Y, Moriyama T, Goto T, Kawada T. 2011. Activation of PPARα suppresses postprandial lipidemia through fatty acid oxidation in enterocytes. *Biochem Biophys Res Commun*. 410:1-6.
15. Uchida A, Slipchenko MN, Cheng JX, Buhman KK. 2011. Fenofibrate, a PPARα agonist, alters triglyceride metabolism in enterocytes of mice. *Biochim Biophys Acta.* 1811:170-6.

16. J.M. Mariadason, C. Nicholas, K.E. L'Italien, M. Zhuang, H.J. Smartt, B.G. Heerdt, W. Yang, G.A. Corner, A.J. Wilson, L. Klampfer, D. Arango, L.H. Augenlicht. 2005. Gene expression profiling of intestinal epithelial cell maturation along the crypt-villus axis. *Gastroenterology.* 128. 1081-1088.
17. N. de Wit, M. Derrien, H. Bosch-Vermeulen, E. Oosterink, S. Keshtkar, C. Duval, J. de Vogel-van den Bosch, M. Kleerebezem, M. Muller, R. van der Meer. 2012. Saturated fat stimulates obesity and hepatic steatosis and affects gut microbiota composition by an enhanced overflow of dietary fat to the distal intestine. *Am. J. Physiol. Gastrointest. Liver Physiol.* 303:G589-G599.
18. Buttet M, Traynard V, Tran TT, Besnard P, Poirier H, Niot I. 2013. From fatty-acid sensing to chylomicron synthesis: Role of intestinal lipid-binding proteins. *Biochimie.* S0300-9084(13)00281-2.
19. Bünger M, van den Bosch HM, van der Meijde J, Kersten S, Hooiveld GJ, Müller M. 2007. Genome-wide analysis of PPARalpha activation in murine small intestine. *Physiol Genomics.* 30:192-204.
20. Suto J, Matsuura S, Imamura K, Yamanaka H, Sekikawa K. 1998. Genetic analysis of non-insulin-dependent diabetes mellitus in KK and KK-Ay mice. *Eur J Endocrinol*. 139: 654-61.
21. M.S. Kang, S. Hirai, T. Goto, K. Kuroyanagi, I.Y. Kim, K. Ohyama, T. Uemura, J.Y. Lee, T. Sakamoto, Y. Ezaki, R. Yu, N. Takahashi, T. Kawada. 2009. Dehydroabietic acid a phytochemical acts as ligand for PPARs in macrophages and adipocytes to regulate inflammation of mouse adipose tissues. *BioFactors.* 35:442–448.
22. Uemura T, Hirai S, Mizoguchi N, Goto T, Lee JY, Taketani K, Nakano Y, Shono J, Hoshino S, Tsuge N, Narukami T, Takahashi N, Kawada T. 2010. Diosgenin present in fenugreek improves glucose metabolism by promoting adipocyte differentiation

and inhibiting inflammation in adipose tissues. *Mol Nutr Food Res*. 54:1596-608.

23. Uchida A, Whitsitt MC, Eustaquio T, Slipchenko MN, Leary JF, Cheng JX. 2012. Reduced triglyceride secretion in response to an acute dietary fat challenge in obese compared to lean mice. *Front Physiol* 3:doi:10.3389/fphys.00026.
24. Masson CJ, Plat J, Mensink RP, Namiot A, Kisielewski W, Namiot Z. 2010. Fatty acid and cholesterol transporter protein expression along the human intestinal tract. *PLoS One*. 5:e10380.
25. Nassir F, Wilson B, Han X, Gross RW, Abumrad NA. 2007. CD36 is important for fatty acid and cholesterol uptake by the proximal but not distal intestine. *J Biol Chem.* 282:19493-501.
26. Schoonjans K, Staels B, Auwerx J. 1996. Role of PPAR in mediating the effect of fibrates and fatty acids on gene expression. *J Lipid Res.* 37:907−25.
27. Goto T, Takahashi N, Taimatsu A, Egawa K, Katoh S, Lee JY, Kim IL, Uemura T, Hirai S, Kobayashi M, Horio F, Kawada T. 2012. Bixin regulates gene expression involved in lipid metabolism in the liver to improve insulin resistance through PPAR-alpha activation. *J Agr Food Chem.* 60:11952-8.
28. Baker, P.W. and G.F. Gibbons. 2000. Effect of dietary fish oil on the sensitivity of hepatic lipid metabolism to regulation by insulin. *J Lipid Res.* 41:719-26.
29. den Boer MAM, Voshol PJ, Kuipers F, Romijn JA, Havekes LM. 2006. Hepatic glucose production is more sensitive to insulin-mediated inhibition than hepatic VLDL-triglyceride production. *Am J Physiol.* 291:E1360-4.

This research was originally published by Elsevier. The definitive version has been published in Obesity Research & Clinical Practice, Volume 7, Issue 5, Pages e353-e360, September 2013, doi:10.1016/j.orcp.2013.05.005.

Chapter 3

Studies on effects of docosahexaenoic acid on intestinal fatty acid oxidation and postprandial hyperlipidemia via PPARα activation

INTRODUCTION

Over the past few decades, the prevalence of metabolic syndrome has markedly increased worldwide, particularly in wealthy, industrialized countries (1). Metabolic syndrome includes multiple factors such as insulin resistance, dyslipidemia, and central obesity and increases the risk of developing serious metabolic disorders such as cardiovascular diseases and type 2 diabetes. Many epidemiological studies, including prospective cohort studies (2-4), cross-sectional studies (5, 6), and case-control studies (7), demonstrate that postprandial hyperlipidemia is an independent risk factor for cardiovascular disease. Therefore, attenuating postprandial hyperlipidemia could be a key factor for preventing cardiovascular diseases.

High intake of dietary fat significantly increases postprandial plasma triacylglyceride (TG) levels. The epithelial cells in the small intestine are constantly exposed to this dietary fat. Therefore, the regulation of lipid metabolism in intestinal epithelial cells could affect postprandial hyperlipidemia. Previous studies have demonstrated that peroxisome proliferator-activated receptor-α (PPARα) is highly expressed in intestinal epithelial cells along the length of the small intestine as well as in the liver, skeletal muscle, and brown fat (8,9). PPARα, which is a nuclear transcriptional factor, regulates lipid metabolism including fatty acid (FA) oxidation (10-11). Synthetic

PPARα agonists, such as fibrates, decrease circulating lipid levels and are commonly used as drugs for the treatment of hyperlipidemia (12). PPARα knockout (PPARα$^{-/-}$) mice showed dyslipidemia (13, 14). Recently, we and others have reported that activation of PPARα in intestinal epithelial cells improves postprandial hyperlipidemia through enhancing FA oxidation (15, 16). PUFAs, such as docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA), are known to lower plasma TG; the mechanism responsible for their hypolipidemic action is thought to be involved in the regulation of TG clearance from circulation and TG synthesis in the liver (17-19). Recent studies have found that PUFAs increase the mRNA expression levels of genes involved in FA oxidation in intestinal epithelial cells (20). However, it is unknown whether dietary lipids, such as DHA could affect the intestinal lipid metabolism, resulting in improvement of postprandial hyperlipidemia.

In this study, we investigated whether DHA improves postprandial hyperlipidemia by altering the lipid metabolism in intestinal epithelial cells. DHA induced FA oxidation in intestinal epithelial cells by activating PPARα, which attenuated postprandial hyperlipidemia by directly reducing TG secretion from intestinal epithelial cells. Furthermore, we confirmed that hepatic lipid metabolism is unlikely to contribute to these effects of DHA. These findings suggest that activating intestinal PPARα by dietary lipids such as DHA may shed light on postprandial hyperlipidemia-induced cardiovascular diseases.

MATERIALS AND METHODS

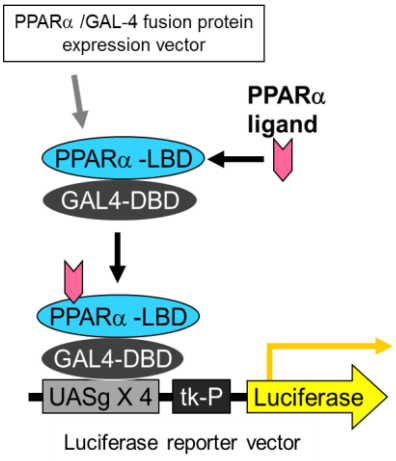
Chemicals and cell culture

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were purchased from Nacalai Tesque (Kyoto, Japan) and dissolved in ethanol. Bezafibrate was purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO) as a stock solution. Decanoic acid (C10) and palmitic acid (C16) were purchased from Nacalai Tesque and Wako Pure Chemicals (Osaka, Japan), respectively. All other chemicals used were from Sigma or Nacalai Tesque and were guaranteed to be reagent or tissue-culture grade.

Human Caco-2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in DMEM (100 mg/dL glucose) containing 10% fetal bovine serum, 1% non-essential amino acid solution, and 10 mg/mL penicillin/streptomycin at 37°C in 5% CO_2/95% air under humidified conditions. Caco-2 cells were seeded at a density of 1.12×10^6 cells/mL on 12-well Transwell® plates (Corning Inc.; Corning, NY, USA) for 2 weeks for differentiation into intestinal epithelial-like cells. To evaluate differentiation of Caco-2 cells, we measured transepithelial electrical resistance (TER). No significance in TER was detected in any experiment (data not shown). The apical medium was changed to DMEM containing either 1 μM or 25 μM DHA or 50 μM Bezafibrate and 600 μM taurocholic acid Na salt hydrate and 500 μM oleic acid. Additionally, the basolateral medium was changed to serum-free DMEM. After 48 h, the basolateral medium was collected to measure TG and apolipoprotein B (apoB) secretion. Cell viability was measured in Caco-2 cells treated with DHA and Bezafibrate based on cell titers (Promega; Fitchburg, WI, USA).

Luciferase assays

Luciferase assays were performed using the modified dual luciferase system as previously described (21). Briefly, for luciferase assays using the GAL4/PPAR chimera system, CV-1 cells or Caco-2 cells were transfected with p4xUASg-tk-luc (reporter plasmid), pM-h PPARα (chimeric plasmid expressing GAL4 DNA-binding domain and human PPARα-ligand binding domain), pM-h PPARγ or pM-h PPARδ, and pRL-CMV (internal control plasmid for normalizing transfection efficiencies). Transfected cells were treated with DHA and EPA, C10 or C16 at the indicated concentrations for 24 h. Bezafibrate (50 μM), pioglitazone (1 μM) or GW501516 (1 μM) were used as positive controls. For luciferase assays using a PPAR full-length system, a reporter plasmid (p4xPPRE-tk-luc) and pRL-CMV were transfected into Caco-2 cells. Transfection was performed using Lipofectamine 2000 (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's protocol. Four hours after transfection, transfected cells were cultured in medium containing fatty acids or positive controls for an additional 24 h, respectively. Luciferase assays were performed using the dual luciferase system according to the manufacturer's protocol.



LBD: ligand binding domain
DBD: DNA binding domain
UASg: Upstream Activating Sequences for galactose
tk-P: thymidine kinase promotor

Gene expression

Total RNA samples were prepared from Caco-2 cells, mouse intestinal epithelial cells, and hepatocytes using Sepasol Super-I (Nacalai Tesque) and Qiazol Lysis reagent (Qiagen; Hilden, Germany) according to the manufacturer's instructions, respectively.

Using M-MLV reverse transcriptase (Invitrogen), total RNA was reverse-transcribed following the manufacturer's protocol using a thermal cycler (Takara; Shiga, Japan). To quantify mRNA expression, real-time PCR was performed using a LightCycler System (Roche Diagnostics; Mannheim, Germany) using SYBR Green fluorescence signals as described previously (22). Oligonucleotide primers of human and mouse *36B4* and *Pparα* target genes used in this study were designed using a PCR primer selection program found in the website of the Virtual Genomic Center from the GenBank database, as previously described (Table 1). To compare mRNA expression levels among samples, copy numbers of all transcripts were divided by that of human and mouse *36B4*, showing a constant expression level. All mRNA expression levels are represented relative to the control in each experiment.

Table 1 Primers used for quantitative real-time PCR

Gene	Forward primer	Reverse primer
human *36b4*	AAACTGCTGCCTCATATCCGG	TTGTAGATGCTGCCATTGTCGA
human *Acs*	CTGCTGTTTTCGCTGGGTCC	AGCAGAGCTTCGCAGCGGC
human *Cpt1a*	AGGCAGAAGAGGTGACGATCG	AATCATCAAGAAATGTCGCACGA
human *Aox*	AAGCAACAGCATCTGAGCGAAT	GGCATGGTGTCCTATTTGAACG
human *Ucp2*	CAAATGAGCTTTGCCTCTGTC	ATGGTCTTGTAGGCATTGACG
human *Fabp*	CCTTCATGAAGGCAATCGGTC	AATGTCATGGTATTGGTGATTATGTCG
mouse *36b4*	CTTTGGCGGGATTAGTCGAAG	TGTGTGTCTGCAGATCGGGTAC
mouse *Acs*	GAGATATTCTGGCCACCGATCA	ACGTATCCCTGGACTAGGACCG
mouse *Cpt1a*	GGCCTCTGTGGTACACGACAA	CTCAGTGGGAGCGACTCTTCA
mouse *Aox*	CAGGATCCGACTGTTTACC	CTTGTTCGCGCAAGTGAGG
mouse *Ucp2*	CCCAGCCTACAGATGTGGTAA	GAGGTTGGCTTTCAGGAGAGT
mouse *Fabp*	AAGACAGCTCCTCCTCGAAGGTT	TGACCAAATCCCCTAAATGCG
mouse *Cd36*	ATTGTACCTGGGAGTTGGCGAGAA	AACTGTCTGTAGACAGTGGTGCCT

Oxygen consumption rate (OCR) in Caco-2 cells

The cellular oxygen consumption rate (OCR) was measured using a Seahorse Bioscience XF analyzer in 24-well plates at 37°C, with correction for positional temperature variations adjusted for the four empty wells in the plate (23, 24). Caco-2

cells were cultured for 2 weeks after seeding on the plate and were treated with PPARα agonist, 50 μM Bezafibrate, or either 1 μM or 25 μM DHA for 24 h. Immediately before the measurement, cells were washed, and 675 μL of non-buffered (sodium-carbonate-free) pH 7.4 DMEM medium supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 1.9 g/L NaCl, and 25 mM glucose was added to each well. After equilibration for 30 min, 2-min measurements were performed at 3-min intervals with inter-measurement mixing to homogenize the oxygen in the medium. OCR (pmol/min) was divided by protein amount in each well.

TG and apolipoprotein B (apoB) secretion in Caco-2 cells

To measure TG secretion, we used the triglyceride E Test WAKO (Wako Pure Chemicals). To measure apolipoprotein B (apoB) secretion, an enzyme-linked immunosorbent assay (ELISA) was performed using an anti-human low-density lipoprotein apo B antibody (Clone 12G10; Monosan; Uden, Netherlands), affinity-purified anti-apolipoprotein B (Rockland; Gilbertsville, PA, USA), and horse radish peroxidase (HRP)-conjugated anti-goat IgG (Promega) as the capture, primary, and secondary antibodies, respectively. Details of these procedures have been previously described (15). HRP activity was detected using a 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (KPL; Gaithersburg, MD, USA).

Animal experiments

DHA-rich oil containing 25.4% DHA and 8% EPA was a gift from NOF CORPORATION (Kanagawa, Japan). EPA-rich oil containing 28.4% EPA and 12.3%

DHA was a gift from Nippon Suisan Kaisha, Ltd. (Tokyo, Japan). All other chemicals were from Sigma or Nacalai Tesque and were guaranteed to be reagent or tissue-culture grade.

All mice were maintained separately in a temperature-controlled (23°C) facility under a constant 12 h light/dark cycle with free access to water. To analyze the effects of DHA on intestinal lipid metabolism and postprandial hyperlipidemia, 9-week-old male C57BL/6 mice (CLEA Japan; Tokyo, Japan) were fed a high-fat diet (HFD) consisting of 60% (kcal%) fat from dietary oil, 26% protein, and 14% carbohydrate for 1 week to induce postprandial hyperlipidemia (25), and were then divided into three groups with the same average serum TG level and body weight after 16 h fasting. Ten-week-old male C57BL/6 mice were maintained for 1 week either on a 60% HFD or on a diet containing 1.9% DHA or 3.7% DHA, maintaining the total amount of fat at 60%. The detailed composition of the experimental diets is described in Table 2 (26).

Table 2 Composition of experimental diets

Experimental diet	Control	1.9% DHA	3.7% DHA
DHA rich oil (%[b])	0	4.13	8.25
Corn oil (%)	33.5	29.38	25.25
Casein (%)	29	29	29
Sucrose (%)	23.29	23.29	23.29
Vitamin mix (%)	1.45	1.45	1.45
Mineral mix (%)	5.08	5.08	5.08
Cellulose powder (%)	7.25	7.25	7.25
L-Cysteine (%)	0.44	0.44	0.44
Fat energy (en %) [a]	60.5	60.6	60.7
DHA (en %) [a]	0	1.85	3.69
EPA (en %) [a]	0	0.58	1.16

DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid.
[a] Percent of energy from total energy intake
[b] Percent of weight from total food weight

To compare the effects of EPA to those of DHA, EPA-rich oil containing 28.4% EPA and 12.3% DHA was diluted with corn oil to prepare an HFD with final concentrations of 3.4% EPA and 1.5% DHA, maintaining the total amount of fat at 60%, for experiments (Fig. 7). The energy intake of all mice was adjusted by pair feeding, and food intake was determined daily for seven consecutive days. Anesthesia was induced using sevoflurane in all experiments. The procedures for animal care were approved by

the Animal Research Committee of Kyoto University.

To clarify whether the effects of DHA-rich oil on intestinal lipid metabolism and postprandial hyperlipidemia involves PPARα, we used PPARα$^{-/-}$ mice with a C57BL/6 genetic background. PPARα$^{-/-}$ mice were fed HFD consisting of 60% (kcal%) fat for 1 week, and were then divided into two groups with the same average serum TG level and body weight after 16 h fasting. Ten-week-old male PPARα$^{-/-}$ mice were maintained for 1 week either on a 60% HFD or on a 60% HFD containing 3.7% DHA.

For RNA analysis, the proximal intestine and the liver were harvested from the mice. After washing, intestinal epithelial cells were collected using a slide glass. Collected tissue was stored in RNAlater (Ambion; Austin TX, USA; Applied Biosystems, Foster City, CA, USA) at -80°C until use.

Fatty acid oxidation in intestinal epithelial cells and hepatocytes

Fatty acid (FA) oxidation with isolated intestinal epithelial cells and hepatocytes were analyzed as previously described (15, 24). Briefly, collected intestinal epithelial cells and hepatocytes were washed with 1% FBS/DMEM three times and used for experiments. Cells were incubated with a piece of filter paper containing 200 μL 3 N NaOH in DMEM containing 200 μM palmitic acid, 0.01% FA-free BSA, 200 μM l-carnitine, and [^{14}C] palmitic acid (1 μCi /mL) (American Radiolabeled Chemicals; St. Louis, MO, USA) at 37°C for 2 h. The tubes were gently shaken every 30 min during incubation. After 2 h of incubation, 200 μL of 12 N HCl was added to the cells to release [^{14}C]-CO_2 and incubated at 37°C overnight to trap [^{14}C]-CO_2. The saturated filter paper containing trapped [^{14}C]-CO_2 was assessed for radioactivity in a liquid scintillation counter (LS6500, Beckman Coulter; Brea, CA, USA). The acidified

medium was centrifuged, and 200 μL of supernatant was assessed to determine the amount of [^{14}C]-labeled acid-soluble metabolites, which includes ketone bodies. Protein concentration was determined using a Protein Assay Kit (Bio-Rad; Hercules, CA, USA).

Quantifying DHA in the small intestine and the liver with LC-MS

LC-MS was performed with an Acquity UPLC system coupled to a Xevo Quadrupole Time-of-Flight (QTOF)-MS system (Waters, Milford, MA), equipped with an electrospray source operated in the negative-ion mode with a lock-spray interface for accurate mass measurement. Leucine enkephaline was employed as the lock-mass compound. It was infused straight into the MS system at a flow rate of 20 μL/min at a concentration of 200 pg/μL in 50% acetonitrile and 0.1% formic acid. The data were acquired by using MassLynx software (Waters). External mass was calibrated by following the manufacture's protocol. An aliquot of the extracted sample (3 μL) was injected into an Acquity UPLC BEH-C18 reversed phase column (2.1×100 mm column size, 1.7 μL particle size). The column temperature was set at 40℃. The amount of DHA was estimated from calibration curve obtained by analytical-grade standard compounds. The peak area of m/z [M-H]±0.05 Da was divided by the area of the internal standard (27).

Postprandial TG and apoB secretion

To measure plasma TG concentration, mice were administrated an oral gavage of 300 μL olive oil after a 16-h fast, and blood samples were collected every 30 min to 240 min after olive oil administration from the tail vein of non-anesthetized mice.

To measure TG secretion from intestinal epithelial cells, mice were injected with 500 mg/kg body weight Tyloxapol (T0307, Sigma) into the intraperitoneal cavity to block serum lipase activity after a 16-h fast (28). After 30 min, mice were administrated an oral gavage of 300 μL olive oil. Blood samples were obtained before Tyloxapol injection and every 30 min for 240 min after olive oil administration. Plasma TG concentration was determined using the triglyceride E Test Wako kit (Wako Pure Chemicals).

To measure postprandial apoB48 secretion, plasma collected at 120 min was mixed with Laemmli Sample buffer (Bio-Rad) (1:8) and boiled for 5 min at 95°C. Plasma samples were subjected to SDS-PAGE on a 5% gel. Separated proteins were transferred electrophoretically to polyvinylidene fluoride membranes (Millipore Corporation, Billerica, MA, USA), which were blocked with 5% non-fat dried milk in phosphate-buffered saline. The membranes were incubated with the anti-mouse apoB48/100 antibodies (Meridian Life Science, Memphis, TN, USA), and then with peroxidase-conjugated anti-rabbit IgG antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), respectively. Protein bands were detected using an enhanced chemiluminescence (ECL) Western blotting detection system (Millipore Corporation). The bands were quantitatively evaluated using National Institutes of Health (NIH) Image J software.

TG accumulation in intestinal epithelial cells of mice

Lipids in intestinal mucosa were extracted using the hexane/isopropanol (3:2) extraction methods (29). Briefly, intestinal mucosa was homogenized using hexane/isopropanol (3/2) for 1 min, the suspension was centrifuged, and the pellet was

rinsed with the same solvent. The entire liquid phase was evaporated, the dried extract dissolved in isopropanol, and TG content was measured as above. Triolein dissolved in isopropanol was used as the standard for TG. The efficiency of extraction was measured by comparing the recovery of triolein in samples that had been spiked and samples that had not been spiked with known quantities of triolein standard. The assessed recovery was 81.2 ± 4.7%.

TG amounts in feces of mice

The feces were dried at 60°C overnight and the lipids were extracted using the Folch method (30). This analysis enables measurement of lipids extracted per gram of dried fecal samples. Briefly, lipids present in the feces were extracted using chloroform/methanol (2:1), dissolved in isopropanol, and TG content was measured as above.

Statistical analysis

Data are presented as means ±S.E.M. For analyses of two groups, unpaired Student's *t*-test was used. To analyze three or more groups, analysis of variance (ANOVA) was used along with Tukey-Kramer's multiple comparison tests to determine statistical significance. Differences were considered significant at $P < 0.05$.

RESULTS

DHA activated PPARα in CV-1cells and Caco-2 cells

First, we investigated whether DHA activated PPARα based on a luciferase assay using the GAL4/PPARα chimera system. DHA activated luciferase activity of PPARα in CV-1 cells in a dose-dependent manner (Fig. 1A). Furthermore, DHA stimulated PPAR-response element (PPRE)-luciferase activity in Caco-2 cells (Fig. 1B). DHA also activated luciferase activity of PPARα in Caco-2 cells (Fig. 1C). The effects of DHA on PPARα activation were higher than those of EPA under our experimental conditions (approximately 5.9- and 2.6-fold increases at 25 μM DHA and EPA, respectively), as shown in Fig. 1C. Moreover, DHA enhanced the activation of PPARγ by approximately 1.7-fold (Fig. 1D). However, DHA did not increase PPARδ activation in Caco-2 cells (Fig. 1E and F). DHA showed the higher activation of PPARα than other fatty acids, such as decanoic acid (C10) and palmitic acid (C16) in Caco-2 cells, although C16 activated PPARα (Fig. 1G). Cytotoxicity was not observed following 25 μM DHA treatment of Caco-2 cells (data not shown). These results suggest that DHA induces PPARα activation in intact cells.

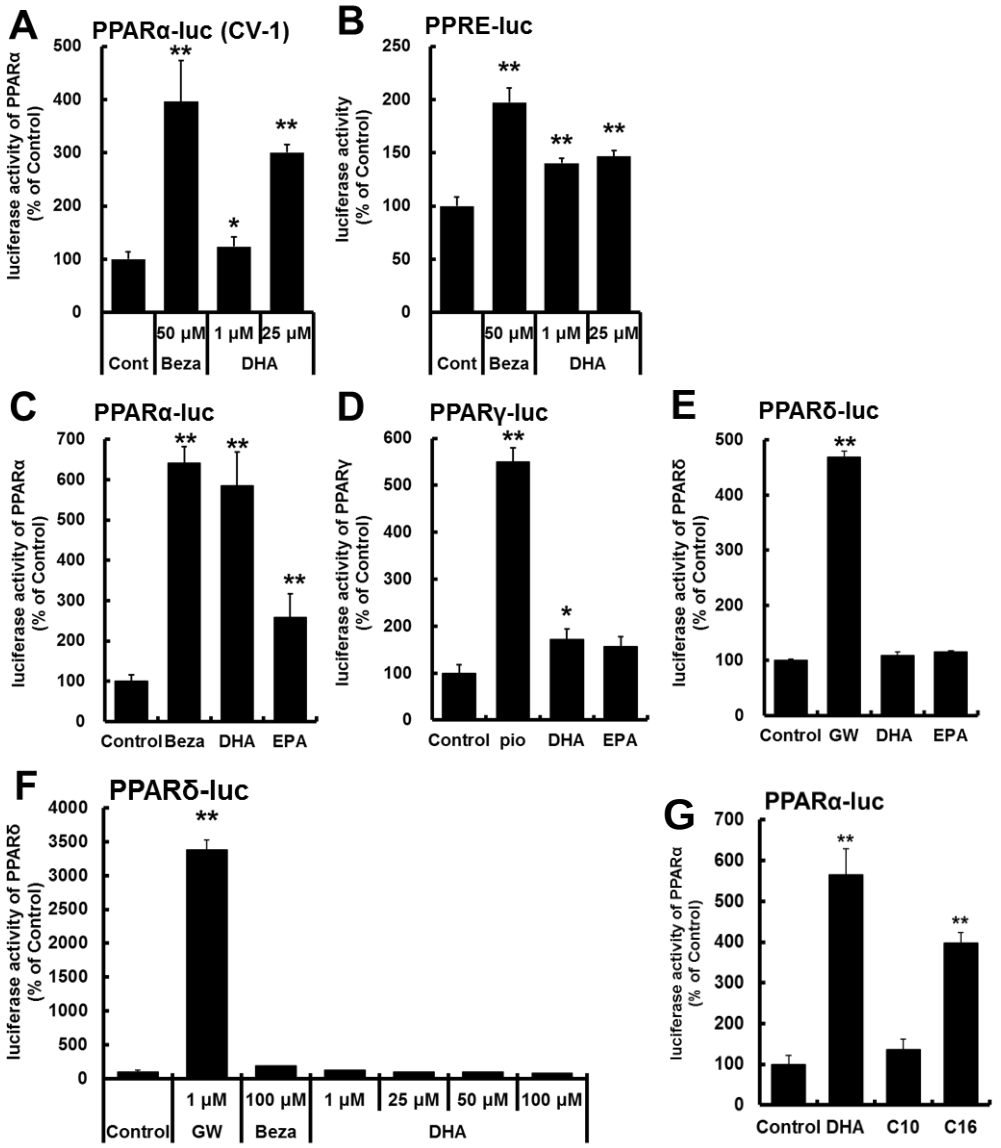


Fig. 1 DHA activated PPARα in luciferase assays using the GAL4 chimera and PPRE-luc systems

(A) Luciferase assay using the GAL4/ PPARα chimera system in CV-1 cells. Reporter plasmids (p4XUASg-luc and pRL-CMV) were transfected into CV-1 cells together with pM-h PPARα. Transfected cells were treated with Bezafibrate or DHA at the indicated concentrations for 24 h. (B) Luciferase assay using a PPRE-luc system in Caco-2 cells. Reporter plasmids (p4XPPRE-tk-luc and pRL-CMV) were transfected into Caco-2 cells. Transfected cells were treated with Bezafibrate or DHA at the indicated concentrations for 24 h. (C-E) Luciferase assay using the GAL4/ PPARα, PPARγ and PPARδ chimera system in Caco-2 cells. Reporter plasmids were transfected with pM-hPPARα, pM-hPPARγ and pM-hPPARδ in Caco-2 cells. Transfected cells were treated with 25 μM DHA and EPA, and 50 μM Bezafibrate, 1 μM pioglitazone or 1 μM GW501516 as positive controls for 24 h. (F) Luciferase assay using the GAL4/ PPARδ chimera system in Caco-2 cells. Reporter plasmid was transfected with pM-hPPARδ in Caco-2 cells. Transfected cells were treated with 1-100 μM DHA, 1 μM GW501516 and 100 μM Bezafibrate as positive controls for 24 h. (G) Luciferase assay using the GAL4/ PPARα chimera system in Caco-2 cells transfected with reporter plasmid and pM-hPPARα. Transfected cells were treated with 25 μM DHA, decanoic acid (C10) and palmitic acid (C16) for 24 h. The average activity of luciferase in a vehicle control was set at 100% and the relative activities were represented as fold induction relative to that of control. Values are means ± S.E.M. of 5-10 tests. *$P < 0.05$ and **$P < 0.01$ compared to each control.

DHA induced the genes involved in FA oxidation and OCR in Caco-2 cells

To investigate the effects of PPARα activation by DHA on intestinal lipid metabolism, we measured mRNA expression levels of genes involved in FA oxidation in DHA-treated Caco-2 cells. DHA induced mRNA expression of genes involved in FA oxidation, such as *acyl-CoA synthase* (*Acs*), *carnitine-palmitoyl-transferase-1a* (*Cpt1a*), *acyl-CoA oxidase* (*Aox*) and *uncoupling protein-2* (*Ucp2*) and other PPARα target genes such as *fatty acid binding protein* (*Fabp*) and *microsomal triglyceride transfer protein (Mtp)* (Fig. 2A–F). Moreover, the oxygen consumption rate (OCR), determined using extracellular flux analysis, was enhanced following DHA treatment (Fig. 2G). In contrast, decanoic acid (C10) with little activity toward PPARα, did not affect mRNA expression of *Cpt1a*, and palmitic acid (C16) with lower PPARα activity than DHA, did not significantly induce *Cpt1a* expression in Caco-2 cells (Fig. 1G and 2H). These findings suggest that DHA enhances FA oxidation in Caco-2 cells.

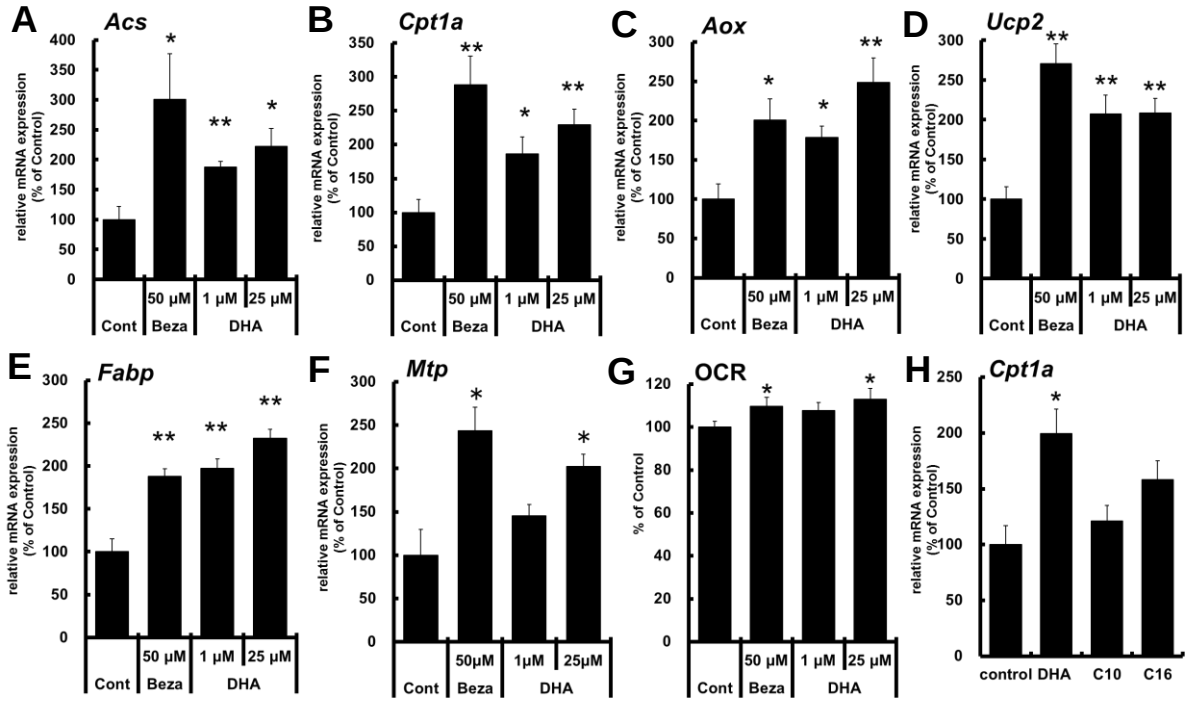


Fig. 2 FA oxidation-related gene expressions and oxygen consumption rate (OCR) were increased in DHA-treated Caco-2 cells

(A–F) The mRNA expression levels of FA oxidation-related genes (*Acs, Cpt1a,Aox and Ucp2*) and other PPARα target genes (*Fabp* and *Mtp*) were quantified in DHA-treated Caco-2 cells for 24 h. (G) Oxygen consumption rate (OCR) was determined using extracellular flux analysis as described in the "MATERIALS AND METHODS" section. (H) *Cpt1a* expression levels in DHA-, C10- and C16- treated Caco-2 cells. Values of controls were set at 100% and the relative values were represented as fold induction relative to that of control. Values are means ± S.E.M. of 6 tests. *$P < 0.05$ and **$P < 0.01$ compared to each control.

DHA decreased the secretion of TG and apoB from Caco-2 cells

To determine the effects of PPARα activation by DHA on lipid secretion from Caco-2 cells, we examined the amounts of lipid secreted from DHA-treated Caco-2 cells. TG secretion from DHA-treated Caco-2 cells was significantly decreased (to 77% and 72% with either 1 or 25 μM DHA treatment, respectively), as shown in Fig. 3A. DHA treatment reduced the secretion of apoB, which is the primary apolipoprotein of chylomicrons, to 67% and 59% with either 1 or 25 μM DHA treatment, respectively (Fig. 3B). The effects of DHA on secretion were similar to those of Bezafibrate, a potent PPARα agonist (Fig. 3A and B). While C10 did not inhibit TG secretion, C16 did decrease TG secretion from Caco-2 cells. However, the effect of C16 on decrease of TG secretion was lower than that of DHA (Fig. 3C). These results suggest that lipid secretion from intestinal epithelial cells is related to PPARα activity.

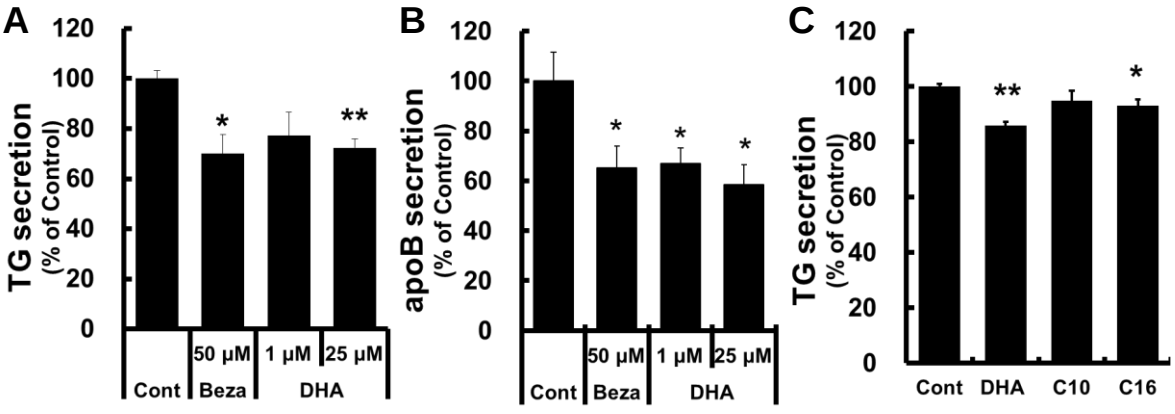


Fig. 3 DHA decreased TG and apoB secretion from Caco-2 cells

TG (A), apoB (B) secretion in DHA-treated Caco-2 cells and TG (C) secretion in DHA-, C10- and C16-treated Caco-2 cells were measured as described in the "MATERIALS AND METHODS" section. Control values were set at 100% and the relative values were represented as fold induction relative to that of control. Values are means ± S.E.M. of 6 mice. *P < 0.05 and **P < 0.01 compared to each control.

DHA-rich oil enhanced FA oxidation in intestinal epithelial cells of C57BL/6 mice

Next, we examined whether the effects of DHA *in vitro* also occurred *in vivo*. Since PPARα agonists are known to reduce food intake in rodents (32), all mice were housed in pair-fed conditions in each experiment; there was no difference in food intake between groups. The mRNA expression levels of FA oxidation-related genes such as *Acs, Cpt1a, Aox and Ucp2*, *cytochrome c (Cyt c)* and other PPARα target genes such as *Fabp* and *Cd36* were increased in C57BL/6 mice fed high-fat diet (HFD) containing DHA-rich oil for one week (Fig. 4A–G). When the cells were incubated with [^{14}C] palmitic acid for 2 h, oxidation of [^{14}C] palmitic acid to CO_2 and acid-soluble metabolites (ASM) were enhanced in intestinal epithelial cells of DHA-rich oil-fed mice compared to control (Fig. 4H and I). Amount of DHA was enhanced in intestinal epithelial cells of DHA-rich oil-fed mice compared to control (Fig. 4J). These results suggest that mitochondrial FA oxidation is enhanced in intestinal epithelial cells of DHA-rich oil-fed mice. However, surprisingly, DHA-rich oil-fed mice showed no increase in mRNA expression levels of FA oxidation-related genes in the liver under the same conditions as shown in Fig. 5A–C. Moreover, the production of CO_2 and ASM were not augmented in the liver of DHA-rich oil-fed mice compared to control mice (Fig. 5D and E). However, amount of DHA was enhanced in the liver of DHA-rich oil-fed mice compared to control (Fig. 5F). These findings suggest that DHA-rich oil enhances FA oxidation in intestinal epithelial cells of mice.

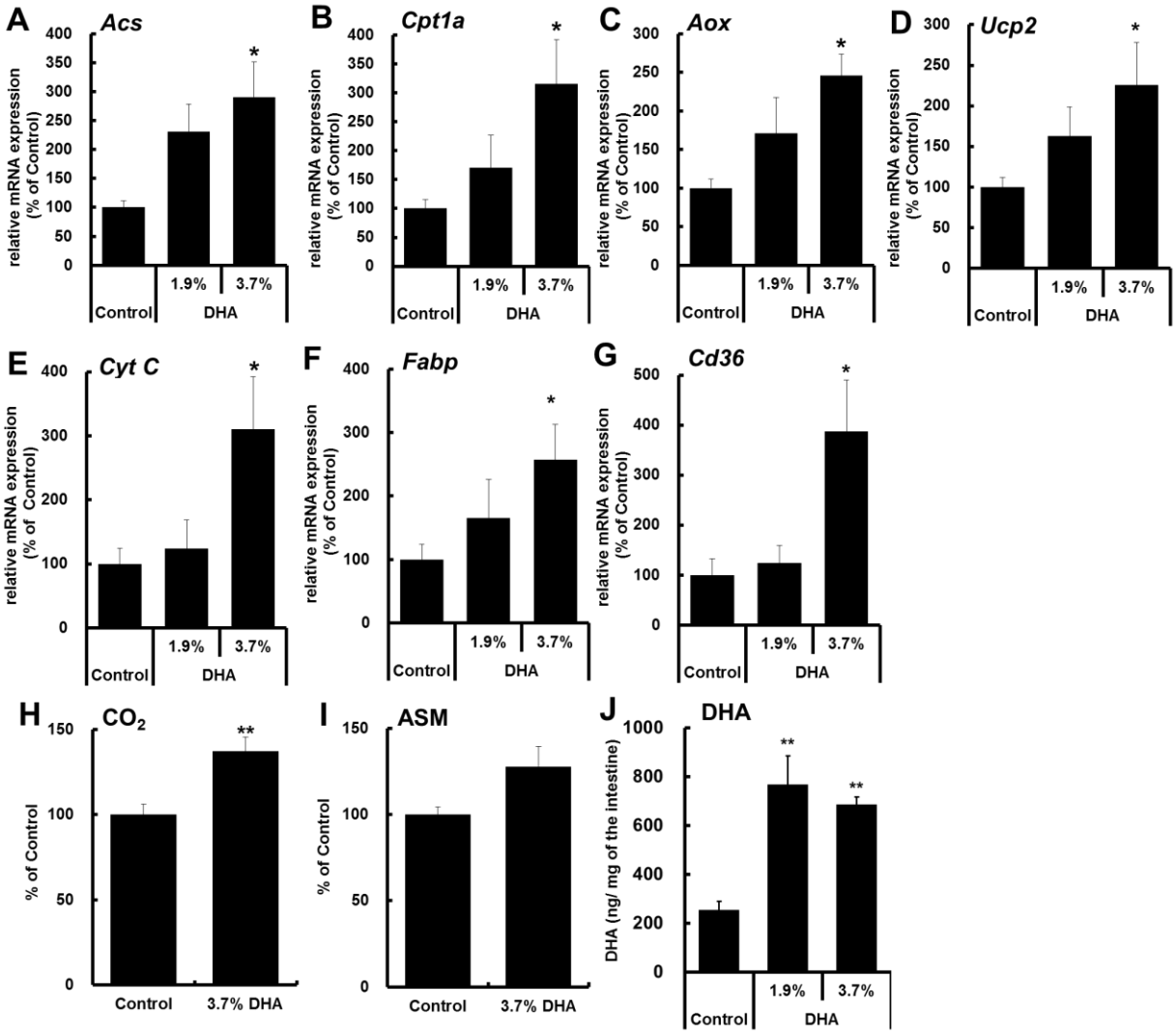


Fig. 4 FA oxidation was enhanced in intestinal epithelial cells of DHA-rich oil fed C57BL/6 mice

(A–G) The mRNA expression levels of FA oxidation-related genes (*Acs*, *Cpt1a*, *Aox* and *Ucp2*), *Cyt c* and other PPARα target genes (*Fabp*, and *Cd36*) in intestinal epithelial cells were quantified. (H and I) Production of CO_2 and ASM in intestinal epithelial cells was determined using [^{14}C] palmitic acid. Control values were set at 100% and the relative values are represented as fold induction relative to that of control. (J) Amount of DHA in the small intestine using LC-MS as described in the "MATERIALS AND METHODS" section. The values are means ± S.E.M. of 6 mice. *P < 0.05 and **P < 0.01 compared to each control.

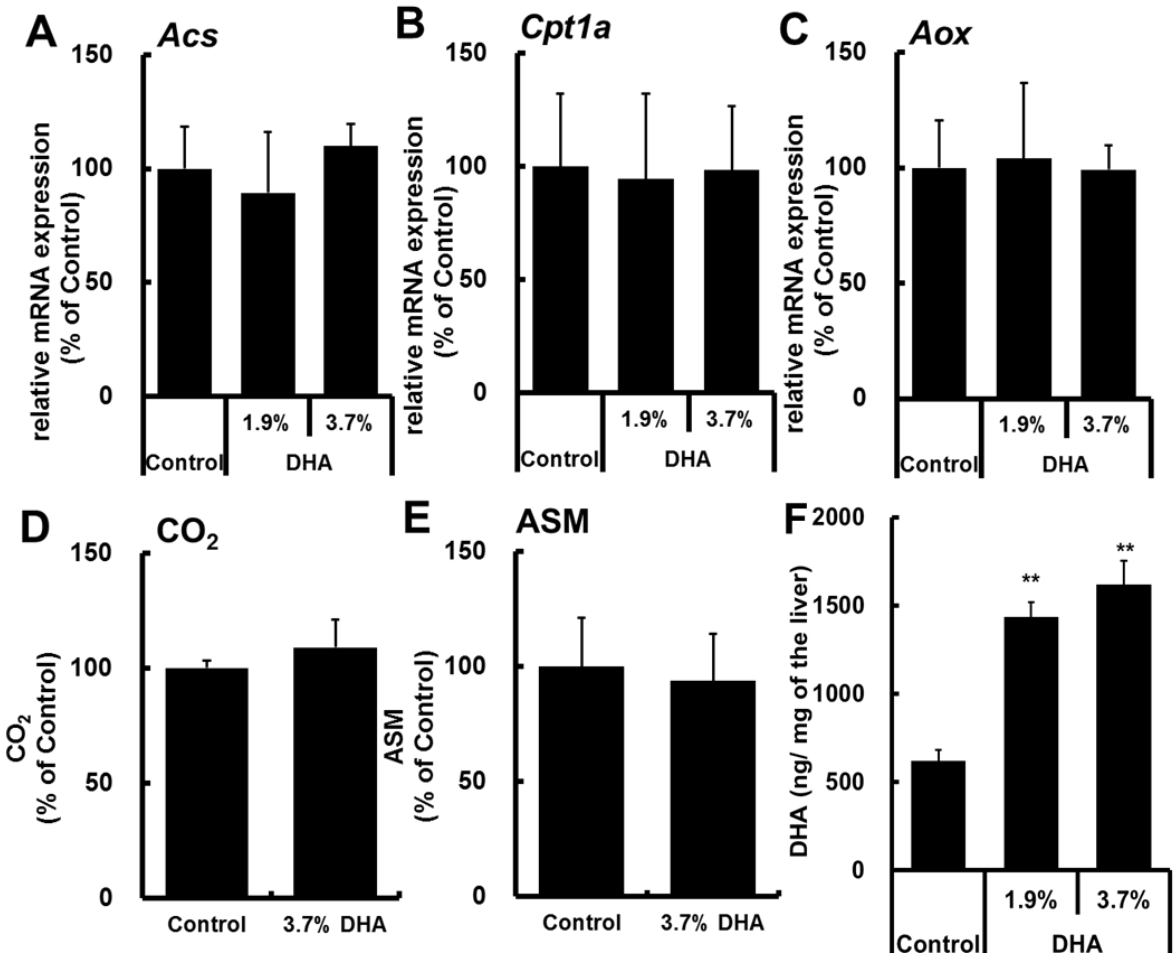


Fig. 5 FA oxidation was not enhanced in the liver of DHA-rich oil fed C57BL/6 mice

(A–C) The mRNA expression levels of FA oxidation-related genes (*Acs*, *Cpt1a* and *Aox*) in the liver were determined by real-time PCR. (D and E) Production of CO_2 and ASM in the liver was determined using [^{14}C] palmitic acid. Control values were set at 100% and the relative values are represented as fold induction relative to that of control. (F) Amount of DHA in the liver using LC-MS as described in the "MATERIALS AND METHODS" section. The values are means ± S.E.M. of 6 tests.

DHA-rich oil attenuated postprandial TG levels via reducing TG secretion from intestinal epithelial cells in C57BL/6 mice

To investigate whether DHA-rich oil decreases postprandial TG levels in mice, we measured plasma TG levels every 30 min to 240 min after oral administration of olive oil. Plasma TG levels were significantly lower in DHA-rich oil-fed mice than those in control from 120 to 240 min after the administration (Fig. 6A). It was also confirmed that postprandial triglyceridemic response determined based on the area under the curve (AUC) was lowered to 66% and 45% in 1.9% and 3.7% DHA-rich oil-fed mice, respectively (Fig. 6B). In addition, plasma apoB48 was also reduced at 120 min in 3.7% DHA-rich oil-fed mice (Fig. 6C). To clarify whether DHA-rich oil altered postprandial TG secretion from intestinal epithelial cells, we measured plasma TG levels after oral administration of olive oil in the presence of Tyloxapol, an inhibitor of TG clearance. Plasma TG levels were significantly decreased after 150 min and AUC was reduced to 66% in 3.7% DHA-rich oil fed mice compared to control (Fig. 6D and E). Furthermore, TG accumulation in intestinal epithelial cells was lower in DHA-rich oil-fed mice than control, and there was no difference in the weight of feces and fecal TG levels between control and DHA-rich oil fed mice (Fig. 6F–H). In contrast, EPA did not affect postprandial lipid metabolism compared to DHA in Caco-2 cells and C57BL/6 mice (Fig. 7). These results suggest that DHA attenuates postprandial hypertriglyceridemia via decreasing TG secretion from intestinal epithelial cells of C57BL/6 mice.

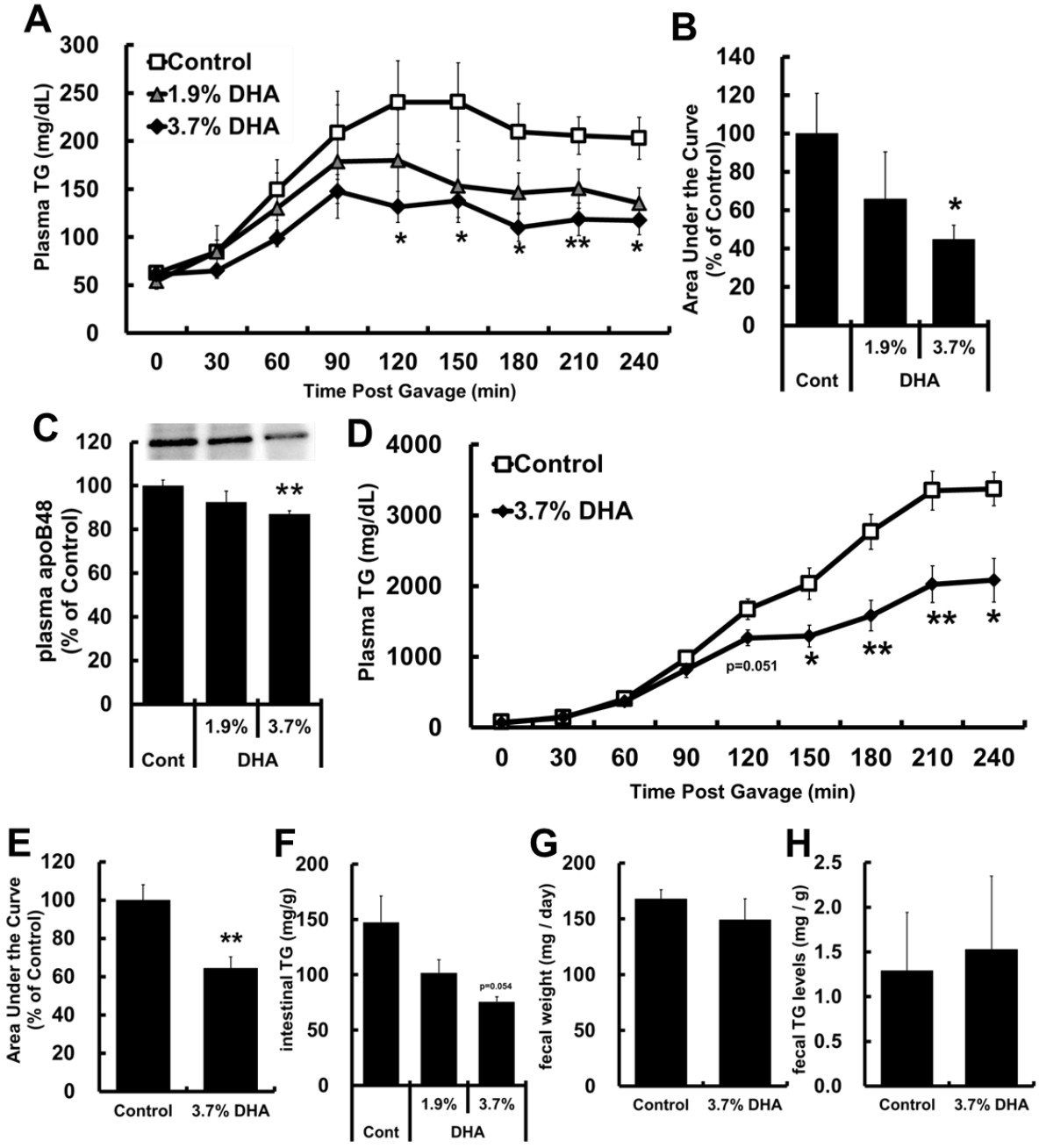


Fig. 6 Postprandial hyperlipidemia was attenuated by decreasing TG secretion from intestinal epithelial cells of DHA-rich oil fed C57BL/6 mice

(A and B) Plasma TG levels every 30 to 240 min and plasma apoB48 levels at 120 min after oral administration of olive oil were measured in control and DHA-rich oil-fed C57BL/6 mice. Plasma TG level was measured by enzymatic colorimetric assay. Plasma apoB48 protein levels at 120 min were visualized by western blotting and band density was determined using National Institutes of Health (NIH) Image J software. (D and E) Postprandial TG secretion in control and DHA-rich oil-fed mice that had been administered Tyloxapol, an inhibitor of TG clearance, were examined. AUC is shown as relative values and is represented as fold induction relative to that of the control, which was set at 100%. (F) TG content in intestinal epithelial cells, (G) the weight of feces and (H) fecal TG levels in control and DHA-rich oil-fed C57BL/6 mice were determined as described in the "MATERIALS AND METHODS" section. The values are means ± S.E.M. of 5–10 mice. *P<0.05 and **P<0.01 compared to each control.

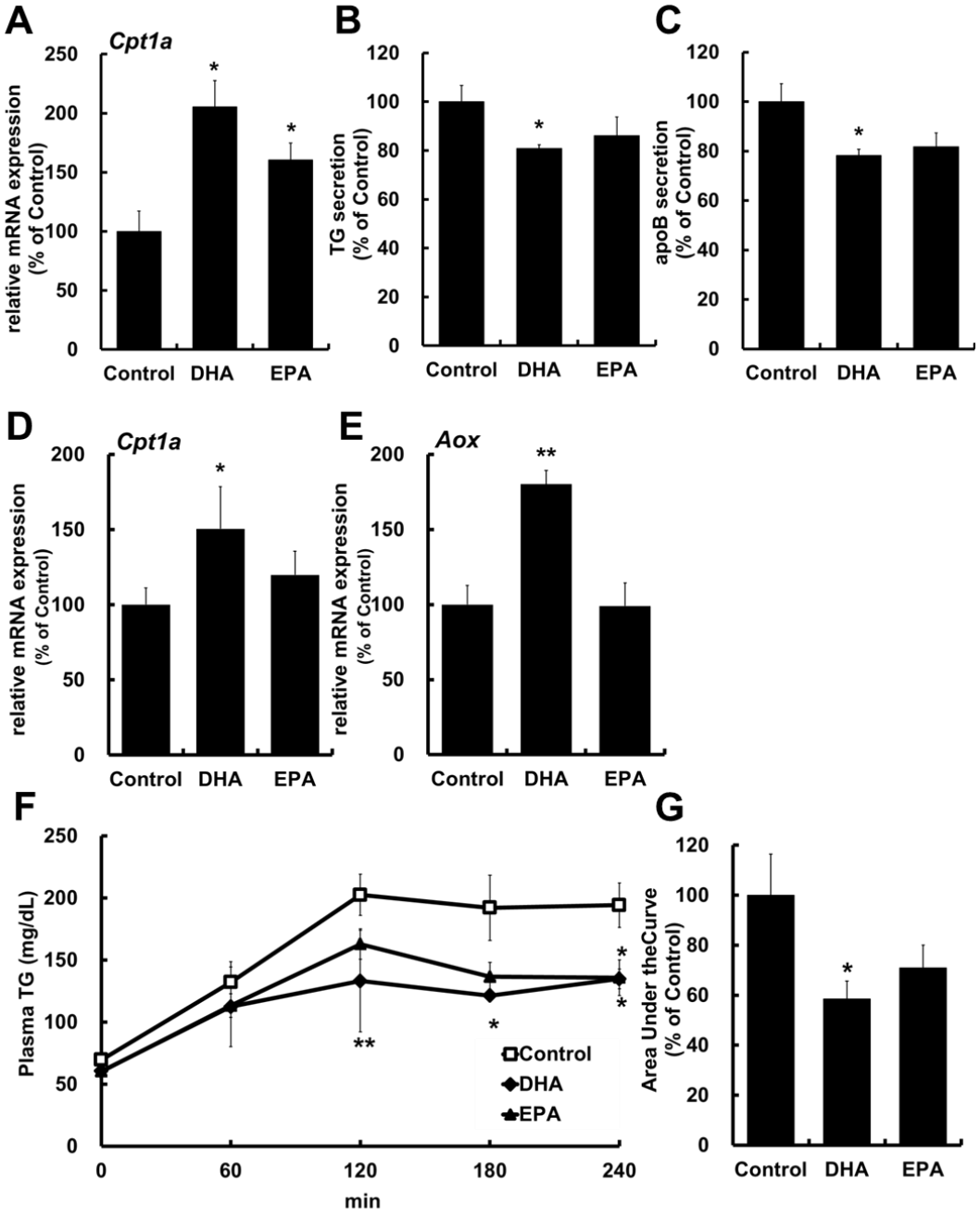


Fig. 7 Effects of eicosapentaenoic acid (EPA) on intestinal lipid metabolism and postprandial hyperlipidemia in C57BL/6 mice

The mRNA expression level of *Cpt1a* (A), TG secretion (B), and apoB secretion (C) were measured in 25 μM EPA-treated Caco-2 cells for 24 h. The mRNA expression levels of *Cpt1a* (D) and *Aox* (E), and plasma TG levels every 60 min to 240 min after oral administration of olive oil (F, G) were investigated in EPA-rich fish oil-fed C57BL/6 mice. Values are the means ± SEM of 6 mice. *P< 0.05 and **P< 0.01 compared to each control.

Effects of DHA on postprandial lipid metabolism were mediated by the activation of intestinal PPARα

To clarify the involvement of PPARα activation in the effects of DHA on postprandial lipid metabolism, we examined the effects of DHA in PPARα$^{-/-}$ mice. The baseline characteristics of PPARα$^{-/-}$ mice compared to control mice are shown in Table 3. The mRNA expression levels of genes involved in FA oxidation (*Acs, Cpt1a*, and *Aox*), and the production of CO_2 and ASM did not change significantly in intestinal epithelial cells of DHA-rich oil-fed PPARα$^{-/-}$ mice (Fig. 8A-E). Moreover, there was no difference in intestinal TG levels between DHA-rich oil-fed PPARα$^{-/-}$ mice and control mice (Fig. 8F). Finally, the effects of DHA-rich oil on plasma TG and apoB levels after olive oil administration were abolished in PPARα$^{-/-}$ mice without and with Tyloxapol (Fig. 8G-I), suggesting that lipid secretion from intestinal epithelial cells is related to PPARα activity. These findings suggest that the activation of intestinal PPARα is a key factor for attenuating postprandial hyperlipidemia via decreasing TG secretion from intestinal epithelial cells.

Table 3

food intake, plasma TG, TG in the liver and the intestine in WT(C57BL/6) and PPARα$^{-/-}$ mice

	WT (C57BL/6)	PPARa$^{-/-}$
food intake (g)	2.28 ± 0.07	2.21 ± 0.12
plasma TG (mg/dL)	125.77 ± 6.52	100.85 ± 5.46 *
TG in the liver (mg/g)	117.88 ± 10.08	82.85 ± 19.04
TG in the intestine(mg/g)	121.73 ± 22.5	74.30 ± 19.04

Values are means ± SEM of 15-18 mice per group

*P<0.05 compared with WT.

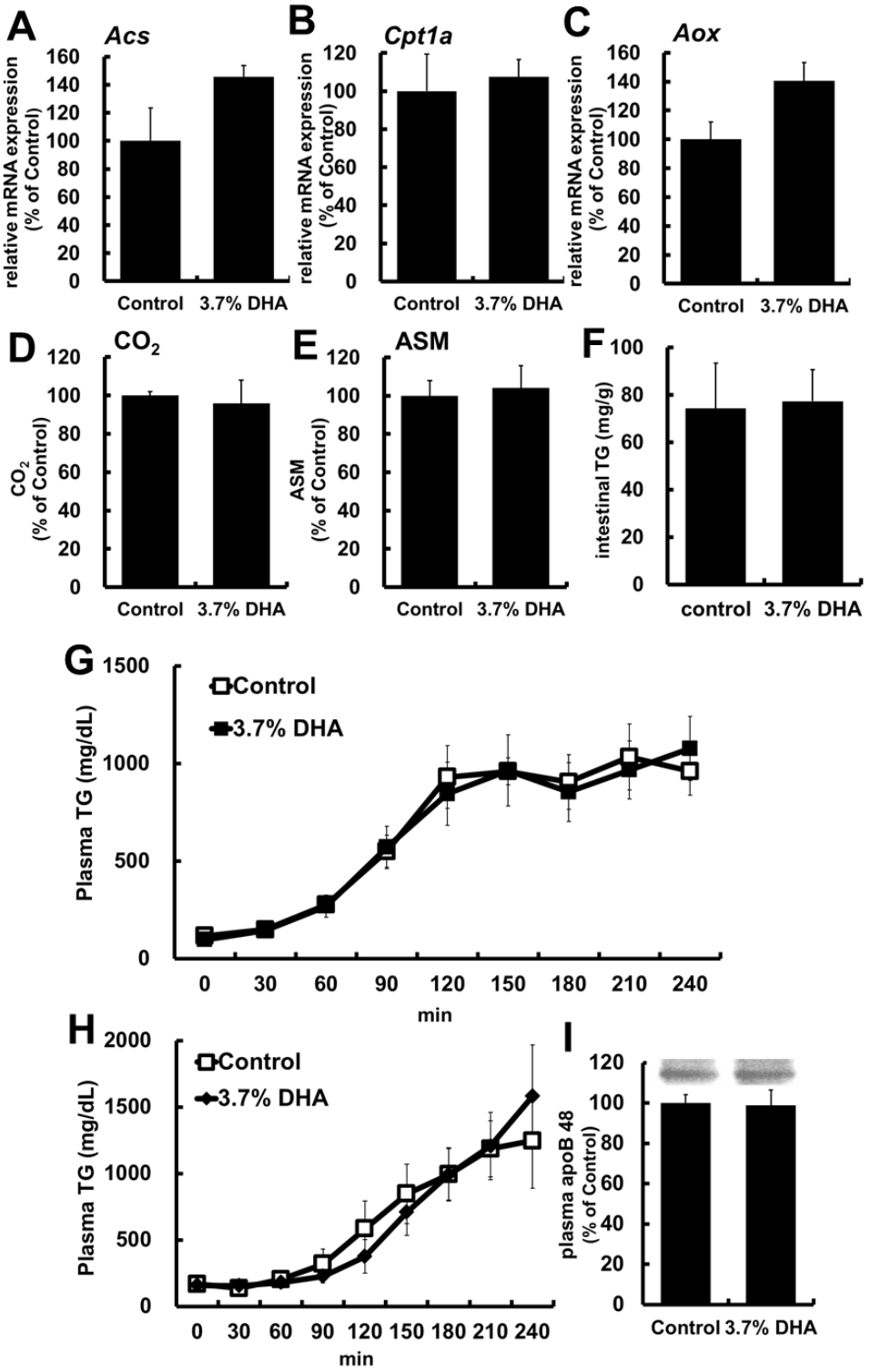


Fig. 8 Effects of DHA-rich oil on intestinal lipid metabolism and postprandial hyperlipidemia were abolished in PPARα$^{-/-}$ mice

(A–C) The mRNA expression levels of FA oxidation-related genes (*Acs*, *Cpt1a*, and *Aox*) in intestinal epithelial cells were determined using real-time PCR. (D and E) CO_2 and ASM production in intestinal epithelial cells of PPARα$^{-/-}$ mice were determined using [^{14}C] palmitic acid. Control values were set at 100% and the relative values are represented as fold induction relative to that of control. (F) TG accumulation in intestinal epithelial cells was measured. (G) Plasma TG levels every 30 min up to 240 min after oral administration of olive oil were measured in control and DHA-rich oil fed PPARα$^{-/-}$ mice. Postprandial TG secretion every 30 up to 240 min (H) and apoB48 secretion at 120 min (I) from intestinal epithelial cells after oral administration of olive oil in control and DHA-rich oil fed PPARα$^{-/-}$ mice which had been administered Tyloxapol, an inhibitor of TG clearance, were examined. Values are means ± S.E.M. of 5 mice.

DISCUSSION

Activation of PPARα is well known to decrease plasma TG levels through FA oxidation in the liver and skeletal muscle (9-11). Although the role of PPARα expressed in intestinal epithelial cells remained obscure (8, 33), we and others have recently demonstrated that PPARα agonists improve postprandial hyperlipidemia through increasing FA oxidation in intestinal epithelial cells (15, 16). It is suggested that PPARα activation reduces TG secretion from intestinal epithelial cells, which attenuates postprandial hyperlipidemia. To clarify the contribution of intestinal PPARα activation to postprandial systemic lipid metabolism, further investigation is necessary, including studies involving intestinal epithelial cell-specific PPARα knockout mice. However, these findings indicate that intestinal PPARα activation plays a critical role in positive regulation of postprandial systemic lipid metabolism.

Although it has been shown that PPARα activation in intestinal epithelial cells reduces postprandial hyperlipidemia, it was unknown whether postprandial hyperlipidemia is also improved by dietary lipids, which generally show lower PPARα activation than synthesized PPARα agonists (34, 35). Previous studies have indicated that DHA increases mRNA expression levels of FA oxidation-related genes in intestinal epithelial cells (36, 37) and that PUFAs including DHA enhance FA oxidation in hepatocytes (38). The present study showed that DHA enhanced FA oxidation and decreased TG secretion in Caco-2 cells and intestinal epithelial cells, resulting in reduction of postprandial hyperlipidemia via PPARα activation in mice. However, surprisingly, no induction of the genes involved in FA oxidation was observed in the liver of DHA rich oil-fed mice under our experimental conditions (Fig. 5). Our findings presented here suggest that effects of DHA on attenuating postprandial hyperlipidemia

are attributed to the decrease of TG secretion from intestinal epithelial cells. During early stages after a meal, most TG secretion into circulation is thought to be derived from dietary fat absorbed in intestinal epithelial cells because they are directly exposed to dietary fat, while insulin prevents hepatic VLDL secretion during the postprandial state (39, 40). In DHA rich oil-fed mice, plasma TG levels were decreased after olive oil administration with Tyloxapol, which inhibits plasma lipoprotein lipase, suggesting that TG secretion from intestinal epithelial cells was reduced (Fig. 6D). This was supported by the results that DHA reduced TG and apoB secretion in Caco-2 cells, as shown in Fig. 3A, B. Moreover, we observed that TG accumulation in intestinal epithelial cells was generally decreased (Fig. 6F) and the level in the weight of feces and fecal TG did not change in DHA rich oil-fed mice (Fig. 6G and H). These findings suggest that DHA is a potent factor to reduce TG secretion from intestinal epithelial cells via FA oxidation by PPARα activation, resulting in attenuating postprandial hyperlipidemia.

In this study, mRNA expression levels of intestinal FA oxidation-related genes in DHA-rich oil-fed PPARα$^{-/-}$ mice were increased, although the increases were not significant (Fig. 8A and C). Previous reports have indicated that PPARδ compensates for the lack of PPARα in the skeletal muscles of PPARα$^{-/-}$ mice (41) and that PPARδ activates FA oxidation (42). DHA and Bezafibrate did not activate PPARδ in our luciferase assays (Fig. 1E and F). However, the concentration of DHA exposed to intestinal epithelial cells may have been much higher than that used in Caco-2 cells. Therefore, the increase in intestinal FA oxidation-related genes in Fig. 8A and C may be related to the PPARδ effect.

The present study showed higher mRNA expression levels of *Cd36* (Fig. 4G), which is involved in FA transport in intestinal epithelial cells of DHA rich oil-fed mice.

Cd36 is thought to be involved in regulating chylomicron production (43, 44). Interestingly, *Cd36* knockout mice showed both fasting and postprandial hyperlipidemia and have been used as a model of postprandial hyperlipidemia (45). A recent study showed that *Cd36* critically regulates FA oxidation in skeletal muscle (46). Additionally, *Cd36* is one of PPARα target genes (47). Therefore, an increase of *Cd36* may contribute to reduction of postprandial hyperlipidemia via intestinal FA oxidation in DHA-rich oil-fed mice.

In conclusion, we found that DHA directly reduced TG secretion from intestinal epithelial cells via activation of PPARα-induced FA oxidation, resulting in improving postprandial hyperlipidemia. The present work suggests that a dietary lipid such as DHA, which activates PPARα, is a promising factor to attenuate postprandial hyperlipidemia via intestinal FA oxidation.

REFERENCES

1. Ogden, C. L., S. Z. Yanovski, M. D. Carroll, and K. M. Flegal. 2007. The epidemiology of obesity. *Gastroenterology.* 132: 2087-102.
2. Bansal, S., J. E. Buring, N. Rifai, S. Mora, F. M. Sacks, and P. M. Ridker. 2007. Fasting compared with nonfasting triglycerides and risk of car diovascular events in women. *JAMA.* 298: 309-16.
3. Freiberg, J. J., A. Tybjaerg-Hansen, J. S. Jensen, and B.G. Nordestgaard. 2008. Nonfasting triglycerides and risk of ischemic stroke in the general population. *JAMA.* 300: 2142-52.
4. Iso, H., Y. Naito, S. Sato, A. Kitamura, T. Okamura, T. Sankai, T. Shimamoto, M.

Iida, and Y. Komachi. 2001. Serum triglycerides and risk of coronary heart disease among Japanese men and women. *Am J Epidemiol.* 153: 490-9.

5. Boquist, S., G. Ruotolo, R. Tang, J. Björkegren, M. G. Bond, U. de Faire, F. Karpe, and A. Hamsten. 1999. Alimentary lipemia, postprandial triglyceride-rich lipoproteins, and common carotid intima-media thickness in healthy, middle-aged men. *Circulation.* 100: 723-8.
6. Patsch, J. R., G. Miesenböck, T. Hopferwieser, V. Mühlberger, E. Knapp, J. K. Dunn, A. M. Gotto, Jr., and W. Patsch. 1992. Relation of triglyceride metabolism and coronary artery disease. Studies in the postprandial state. *Arterioscler Thromb*. 12: 1336-45.
7. Björkegren J, S. Boquist, A. Samnegârd, P. Lundman, P. Tornvall, C. G. Ericsson, and A. Hamsten. 2000. Accumulation of apolipoprotein C-I-rich and cholesterol-rich VLDL remnants during exaggerated postprandial triglyceridemia in normolipidemic patients with coronary artery disease. *Circulation.* 101: 227-30.
8. M. Bunger, H. M. Van Den Bosch, J. Van Der Meijde, S. Kersten, G. Hooiveld, and M. Muller. 2007. Genome-wide analysis of PPARalpha activation in murine small intestine. *Physiol. Genomics*, 30: 192–204
9. Kerste, S., B. Desvergne, and W. Wahli. 2000. Roles of PPARs in health and disease. *Nature.* 405: 421–424.
10. Hashimoto, T., W. Cook, C. Qi, A. Yeldandi, J. Reddy, and M. Rao. 2000. Defect in peroxisome proliferator-activated receptor alpha inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. *J. Biol. Chem.* 275: 28918–28928.
11. Minnich, A., N. Tian, L. Byan, and G. Bilder. 2001. A potent PPAR agonist

stimulates mitochondrial fatty acid-oxidation in liver and skeletal muscle. *Am. J. Physiol. Endocrinol. Metab*. 280: E270–E279.

12. Schoonjans, K., B. Staels, and J. Auwerx. 1996. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *J. Lipid Res.* 37: 907–925.
13. Akiyama, T. E., C. J. Nicol, C. Fievet, B. Staels, J. M. Ward, J. Auwerx, S. S. Lee, F. J. Gonzalez, and J. M. Peters. 2001. Peroxisome proliferator-activated receptor-alpha regulates lipid homeostasis, but is not associated with obesity: studies with congenic mouse lines. *J. Biol. Chem*. 276: 39088–39093.
14. Lindén, D., M. Alsterholm, H. Wennbo, and J. Oscarsson. 2001. PPARalpha deficiency increases secretion and serum levels of apolipoprotein B-containing lipoproteins. *J Lipid Res.* 42:1831-40.
15. Kimura, R, N. Takahashi, K. Murota, Y. Yamada, S. Niiya, N. Kanzaki, Y. Murakami, T. Moriyama, T. Goto, and T. Kawada. 2011. Activation of peroxisome proliferator-activated receptor-α (PPARα) suppresses postprandial lipidemia through fatty acid oxidation in enterocytes. *Biochem. Biophys. Res. Commun.* 410: 1-6.
16. Uchida, A., M. N. Slipchenko, J. X. Cheng, and K. K. Buhman. 2011. Fenofibrate, a peroxisome proliferator-activated receptor α agonist, alters triglyceride metabolism in enterocytes of mice. *Biochim. Biophys. Acta.* 1811: 170-6.
17. Wong, S. H., E. A. Fisher, and J. B. Marsh. 1989. Effects of eicosapentaenoic and docosahexaenoic acids on apoprotein B mRNA and secretion of very low density lipoprotein in HepG2 cells. *Arteriosclerosis.* 9:836-41.
18. Nestel, P.J., W.E. Connor, M.F. Reardon, S. Connor, S. Wong, and R. Boston. 1984.

Suppression by diets rich in fish oil of very low density lipoprotein production in man. *J. Clin. Invest.* 74:82–89.

19. Park, Y., and W.S. Harris, Omega-3 fatty acid supplementation accelerates chylomicron triglyceride clearance. 2003. *J. Lipid Res.* 44:455–463.

20. van Schothorst, E. M., P. Flachs, N. L. Franssen-van Hal, O. Kuda, A. Bunschoten, J. Molthoff, C. Vink, G. J. Hooiveld, J. Kopecky, and J. Keijer. 2009. Induction of lipid oxidation by polyunsaturated fatty acids of marine origin in small intestine of mice fed a high-fat diet. *BMC Genomics.* 10: 110.

21. Goto, T., N. Takahashi, S. Kato, K. Egawa, S. Ebisu, T. Moriyama, T. Fushiki, and T. Kawada. 2005. Phytol directly activates peroxisome proliferator-activated receptor alpha (PPAR alpha) and regulates gene expression involved in lipid metabolism in PPAR alpha-expressing HepG2 hepatocytes. *Biochem. Biophys. Res. Commun.* 337: 440-5.

22. Kuroyanagi, K., M. S. Kang, T. Goto, S. Hirai, K. Ohyama, T. Kusudo, R. Yu, M. Yano, T. Sasaki, N. Takahashi, and T. Kawada. 2008. Citrus auraptene acts as an agonist for PPARs and enhances adiponectin production and MCP-1 reduction in 3T3–L1 adipocytes. *Biochem. Biophys. Res. Commun.* 366: 219–225.

23. Wu, M., A. Neilson, A. L. Swift, R. Moran, J. Tamagnine, D. Parslow, S. Armistead, K. Lemire, J. Orrell, J. Teich, S. Chomicz, and D.A. Ferrick. 2007. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *Am. J. Physiol. Cell Physiol.* 292: C125–C136.

24. Goto, T., J. Y. Lee, A. Teraminami, Y. I. Kim, S. Hirai, T. Uemura, H. Inoue, N. Takahashi, and T. Kawada. 2011. Activation of peroxisome proliferator-activated

receptor-alpha stimulates both differentiation and fatty acid oxidation in adipocytes. *J Lipid Res.* 52: 873-84.

25. Hernández Vallejo, S. J., M. Alqub, S. Luquet, C. Cruciani-Guglielmacci, P. Delerive, J. M. Lobaccaro, A. D. Kalopissis, J. Chambaz, M. Rousset, and J. M. Lacorte. 2009. Short-term adaptation of postprandial lipoprotein secretion and intestinal gene expression to a high-fat diet. *Am J Physiol Gastrointest Liver Physiol*. 296: G782-92.

26. Nakatani, T., H. J. Kim, Y. Kaburagi, K. Yasuda, and O. Ezaki. 2003. A low fish oil inhibits SREBP-1 proteolytic cascade, while a high-fish-oil feeding decreases SREBP-1 mRNA in mice liver: relationship to anti-obesity. *J Lipid Res.* 44:369-79.

27. Takahashi H, Suzuki H, Suda K, Yamazaki Y, Takino A, Kim YI, Goto T, Iijima Y, Aoki K, Shibata D, Takahashi N, Kawada T. 2013. Long-Chain Free Fatty Acid Profiling Analysis by Liquid Chromatography-Mass Spectrometry in Mouse Treated with Peroxisome Proliferator-Activated Receptor α Agonist. *Biosci Biotechnol Biochem.* 77:2288-93.

28. X. Lin, P. Yue, Z. Chen, and G. Schonfeld. 2005. Hepatic triglyceride contents are genetically determined in mice: results of a strain survey. *Am J Physiol Gastrointest Liver Physiol.* 288:G1179-89.

29. Eder, K., A. M. Reichlmayr-Lais, and M. Kirchgessner. 1993. Studies on the extraction of phospholipids from erythrocyte membranes in the rat. *Clin Chim Acta*. 219: 93-104.

30. Perera, M.A., S.Y. Choi, E.S. Wurtele, and B.J. Nikolau. 2009. Quantitative analysis of short-chain acyl-coenzymeAs in plant tissues by LC-MS-MS electrospray ionization method. *J Chromatogr B Analyt Technol Biomed Life Sci.*

877:482-8.

31. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*. 226: 497–509.

32. Fu. J., S. Gaetani, F. Oveisi, J. Lo Verme, A. Serrano, F. Rodríguez De Fonseca, A. H. Rosengarth, Luecke, B. Di Giacomo, G. Tarzia, and D. Piomelli. 2003. Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. *Nature*. 425:90-3.

33. Escher, P., O. Braissant, S. Basu-Modak, L. Michalik, W. Wahli, and B. Desvergne. 2001. Rat PPARs: quantitative analysis in adult rat tissues and regulation in fasting and refeeding. *Endocrinology.* 142:4195-202.

34. Kim, YI., S. Hirai, T. Goto, C. Ohyane, H. Takahashi, T. Tsugane, C. Konishi, T. Fujii, S. Inai, Y. Iijima, K. Aoki, D. Shibata, N. Takahashi, and T. Kawada. 2012. Potent PPARα activator derived from tomato juice, 13-oxo-9,11-octadecadienoic acid, decreases plasma and hepatic triglyceride in obese diabetic mice. *PLoS One.* 7:e31317.

35. Shen. P., M. H. Liu, T. Y. Ng, Y. H. Chan, and E. L. Yong. 2006. .Differential effects of isoflavones, from Astragalus membranaceus and Pueraria thomsonii, on the activation of PPARalpha, PPARgamma, and adipocyte differentiation in vitro. *J Nutr.* 136:899-905.

36. de Vogel-van den Bosch, H.M., M. Bünger, P.J. de Groot, H. Bosch-Vermeulen, G.J. Hooiveld, and M. Müller. 2008. PPARalpha-mediated effects of dietary lipids on intestinal barrier gene expression. *BMC Genomics.* 9:231.

37. Mori, T., H. Kondo, T. Hase, I. Tokimitsu, and T. Murase. 2007. Dietary fish oil

upregulates intestinal lipid metabolism and reduces body weight gain in C57BL/6J mice. *J Nutr.* 137:2629-34.

38. Shearer, G..C., O.V. Savinova, and W.S. Harris. 2012. Fish oil - How does it reduce plasma triglycerides? *Biochim Biophys Acta.* 1821:843-51.

39. Lewis, G.F., K. D. Uffelman, L. W. Szeto, and G.. Steiner. 1993. Effects of acute hyperinsulinemia on VLDL triglyceride and VLDL apoB production in normal weight and obese individuals. *Diabetes.* 42:833-42.

40. Baker, P.W. and G.F. Gibbons. 2000. Effect of dietary fish oil on the sensitivity of hepatic lipid metabolism to regulation by insulin. *J Lipid Res.* 41:719-26.

41. Muoio, D.M., P.S. MacLean, D.B. Lang, S. Li, J.A. Houmard, J.M. Way, D.A. Winegar J.C. Corton, G.L. Dohm, and W.E. Kraus. 2002. Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) alpha knock-out mice. Evidence for compensatory regulation by PPAR delta. *J Biol Chem.* 277:26089-97.

42. Kleiner. S., V. Nguyen-Tran, O. Baré, X. Huang, B. Spiegelman, and Z . Wu. 2009. PPAR{delta} agonism activates fatty acid oxidation via PGC-1{alpha} but does not increase mitochondrial gene expression and function. *J Biol Chem.* 284:18624-33.

43. Drover, V.A., M. Ajmal, F. Nassir, N.O. Davidson, A.M. Nauli, D. Sahoo, P. Tso, and N.A. Abumrad. 2005. CD36 deficiency impairs intestinal lipid secretion and clearance of chylomicrons from the blood. *J Clin Invest.* 115: 1290-7.

44. Tran, T.T., H. Poirier, L. Clément, F. Nassir, M.M. Pelsers, V. Petit, P. Degrace, M.C. Monnot, J.F. Glatz, N.A. Abumrad, P. Besnard, and Niot I. 2011. Luminal lipid regulates CD36 levels and downstream signaling to stimulate chylomicron

synthesis. *J Biol Chem.* 286: 25201-10.

45. Masuda, D., K. Hirano, H. Oku, J.C. Sandoval, R. Kawase, M. Yuasa-Kawase, Y. Yamashita, M. Takada, K. Tsubakio-Yamamoto, Y. Tochino, M. Koseki, F. Matsuura, M. Nishida, T. Kawamoto, M. Ishigami, M. Hori, I. Shimomura, and S. Yamashita. 2009. Chylomicron remnants are increased in the postprandial state in CD36 deficiency. *J Lipid Res.* 50:999-1011.

46. McFarlan, J.T., Y. Yoshida, S.S. Jain, X.X. Han, L.A. Snook, J. Lally, B.K. Smith, J.F. Glatz, J.J. Luiken, R.A. Sayer, A.R. Tupling, A. Chabowski, G.P. Holloway, and A. Bonen. 2012. In vivo, fatty acid translocase (CD36) critically regulates skeletal muscle fuel selection, exercise performance, and training-induced adaptation of fatty acid oxidation. *J Biol Chem.* 287:23502-16.

47. Rakhshandehroo M, B. Knoch, M. Müller, and S. Kersten. 2010. Peroxisome proliferator-activated receptor alpha target genes. *PPAR Res.* 2010: 612089.

This research was originally published in Journal of Lipid Research. Kimura R, Takahashi N, Lin S, Goto T, Murota K, Nakata R, Inoue H, Kawada T. DHA attenuates postprandial hyperlipidemia via activating PPARα in intestinal epithelial cells. *Journal of Lipid Research*. 2013; 54:3258-68.

Summary

Chapter 1

It is well known that activation of peroxisome proliferator-activated receptor (PPAR)-α reduces plasma lipid levels at fasting. Recently, postprandial plasma lipid levels have been found to correlate more closely to cardiovascular diseases than fasting levels. However, the effects of PPARα activation in the small intestine, which is associated with postprandial lipid metabolism, and on postprandial hyperlipidemia have not been clarified. In this study, we examined the effects of PPARα activation on intestinal lipid metabolism and postprandial hyperlipidemia. In Caco-2 cells, Bezafibrate, a potent PPARα agonist, increased mRNA expression levels of fatty acid oxidation-related genes, such as *acyl-CoA oxidase*, *carnitine-palmitoyl-transferase*, and *acyl-CoA synthase*, and oxygen consumption rate (OCR). In addition, it reduced secretion of both triglyceride (TG) and apolipoprotein B (apoB) into the basolateral side. *In vivo* experiments revealed that feeding high-fat diet containing Bezafibrate increased mRNA expression levels of fatty acid oxidation-related genes, and production of CO_2 and acid soluble metabolites in intestinal epithelial cells. Moreover, Bezafibrate treatment decreased plasma TG and apoB levels after oral administration of olive oil in mice. These effects of Bezafibrate were not observed in PPARα knockout mice. These findings indicate that PPARα activation attenuates postprandial hyperlipidemia through enhancement of intestinal fatty acid oxidation, suggesting that intestinal lipid metabolism regulated by PPARα activity is a novel target of PPARα agonist for decreasing circulating levels of lipids under postprandial conditions.

Chapter 2

Postprandial hyperlipidemia is a risk factor for cardiovascular diseases. The small intestine is associated with postprandial lipid metabolism and expresses peroxisome proliferator-activated receptor-α (PPARα), which is a key regulator in the lipid metabolism of peripheral tissues such as the liver and skeletal muscle. It is well known that PPARα activation enhances fatty acid oxidation and decreases circulating lipid level. Recently, we have shown that Bezafibrate, a PPARα,agonist attenuates postprandial hyperlipidemia via enhancing intestinal fatty acid oxidation under physiological conditions. However, it was unknown whether the effects of PPARα activation on postprandial hyperlipidemia were also observed under obese conditions. Here, we found that Bezafibrate enhanced fatty acid oxidation in intestinal epithelial cells of obese diabetic KK-Ay mice. Bezafibrate treatment increased the mRNA expression levels of fatty acid oxidation-related genes, which are target genes of PPARα, and enhanced CO_2 production from [^{14}C]-palmitic acid. Bezafibrate-treated mice showed attenuation of plasma triglyceride level after the oral administration of olive oil. Moreover, the effects of Bezafibrate on fatty acid oxidation were observed in only proximal intestinal epithelial cells. These findings suggest that PPARα activation attenuates postprandial hyperlipidemia via enhancement of fatty acid oxidation in proximal intestine of mice under obese conditions and proximal intestinal PPARα can be a target for prevention of obese-induced postprandial hyperlipidemia.

Chapter 3

It is known that peroxisome proliferator-activated receptor-α (PPARα), whose activation reduces hyperlipidemia, is highly expressed in intestinal epithelial cells. Docosahexaenoic acid (DHA) could improve postprandial hyperlipidemia, however, its relationship with intestinal PPARα activation is not revealed. In this study, we investigated whether DHA can affect postprandial hyperlipidemia by activating intestinal PPARα using Caco-2 cells and C57BL/6 mice. The genes involved in fatty acid (FA) oxidation and oxygen consumption rate were increased, and the secretion of triacylglyceride (TG) and apolipoprotein B (apoB) was decreased in DHA-treated Caco-2 cells. Additionally, intestinal FA oxidation was induced, and TG and apoB secretion from intestinal epithelial cells were reduced, resulting in the attenuation of plasma TG and apoB levels after oral administration of olive oil in DHA-rich oil-fed mice compared to control. However, no increase in genes involved in FA oxidation was observed in the liver. Furthermore, the effects of DHA on intestinal lipid secretion and postprandial hyperlipidemia were abolished in PPARα knockout mice. In conclusion, the present work suggests that DHA can inhibit the secretion of TG from intestinal epithelial cells via PPARα activation, which attenuates postprandial hyperlipidemia.

Acknowledgements

The author would like to express my sincere gratitude to Dr. Teruo Kawada, Professor of Graduate School of Agriculture, Kyoto University, for his continuous supports, hearty encouragement and kindness throughout this study for 7 years.

The author would like to thank Dr. Nobuyuki Takahashi, Assistant Professor of Graduate School of Agriculture, Kyoto University for his advice.

The author would like to express my gratitude to the great teacher, Dr. Tsuyoshi Goto, Assistant Professor of Graduate School of Agriculture, Kyoto University for his meaningful discussion, helpful advices, a lot of kindness and great jokes.

The author would like to express my deepest appreciation to Dr. Shan Lin for her helpful continuous supports, heartfelt encouragement, lovely smile and having precious time together.

The author would like to express my gratitude to Dr. Tomoya Sakamoto for his invaluable advices, helpful suggestions and sincere kindness.

The author would like to express my gratefulness to Dr. Haruya Takahashi for his meaningful discussion and sincere kindness.

The author would like to thank specially to Ms. Mariko Hirata for her helpful supports, hearty kindness and pretty smiles.

The author would like to thank the members of the laboratory of Molecular Function of Food, Graduate School of Agriculture, Kyoto University, for their supports and cheers.

Rino Kimura

List of publications

1. **Kimura R**, Takahashi N, Murota K, Yamada Y, Niiya S, Kanzaki N, Murakami Y, Moriyama T, Goto T, and Kawada T.

   Activation of peroxisome proliferator-activated receptor-α (PPARα) suppresses postprandial lipidemia through fatty acid oxidation in enterocytes

   Biochem Biophys Res Commun, 2011; 410:1-6.

2. **Kimura R**, Takahashi N, Goto T, Murota K, and Kawada T.

   Activation of peroxisome proliferator-activated receptor-α (PPARα) in proximal intestine improves postprandial lipidemia in obese diabetic KK-Ay mice

   Obesity Research & Clinical Practice, 2013; e353-e360.

3. **Kimura R**, Takahashi N, Lin S, Goto T, Murota K, Nakata R, Inoue H, and Kawada T.

   DHA attenuates postprandial hyperlipidemia via activating PPARα in intestinal epithelial cells

   The Journal of Lipid Research, 2013; 54:3258-68.