Fenofibrate Reduces Postprandial Hypertriglyceridemia in CD36 Knockout Mice

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**Aim:** Metabolic syndrome (MetS) and postprandial hypertriglyceridemia (PHTG) are closely related and both are associated with coronary heart disease. We have demonstrated that CD36 deficiency is prevalent in the genetic background of MetS and is accompanied by PHTG concomitantly with an increase in remnants and a decrease in high density lipoprotein cholesterol. These findings make CD36 knockout mice (CD36KO) an interesting model for evaluating PHTG in MetS. Fenofibrate was reported to reduce fasting and postprandial triglyceride (TG) levels in hypertriglyceridemic subjects with MetS. To define its mechanism, we investigated the effect of fenofibrate on PHTG in CD36KO.

**Methods:** Wild-type (WT) and CD36KO mice were fed chow diet and fenofibrate for two weeks. TG concentrations and lipoprotein profiles were assessed during fasting and in the postprandial state in plasma; intestinal mucosa and lymph were collected after oral fat loading for both treatment groups.

**Results:** Fenofibrate treatment markedly suppressed the postprandial TG response in CD36KO along with decreased apoB-48 levels in plasma. HPLC analysis depicted the decrease of TG content in chylomicrons (CM) and CM remnant-sized lipoproteins contributed to this suppression, suggesting that CM and CM remnant production in the intestines might be attenuated by fenofibrate. ApoB-48 and TG levels in intestinal lymph were markedly reduced after treatment. Intestinal mRNA expression of apoB was also reduced in the postprandial state after fenofibrate administration without affecting any other genes related to CM assembly and production.

**Conclusion:** Fenofibrate reduces PHTG in CD36KO partially through attenuating intestinal CM production.

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**Key words:** Fenofibrate, Postprandial hypertriglyceridemia, CD36 knockout mice, Apolipoprotein B-48

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**Introduction**

Metabolic syndrome (MetS), based upon the accumulation of visceral fat, represents a clustering of interrelated risk factors for cardiovascular disease that include abnormally high serum triglyceride (TG) levels in the fasting state\(^1\), \(^2\). Metabolic syndrome presents as a challenge to the healthcare system, particularly due to the increasing prevalence of overweight/obesity and type 2 diabetes mellitus worldwide\(^3\).

The publication of meta-analyses pointing at raised serum TG levels as an independent risk factor for coronary heart disease highly suggests that TG-rich lipoproteins, such as chylomicrons (CM), very low
density lipoproteins (VLDL) and their remnants, are atherogenic.\textsuperscript{4, 5} Triglycerides are routinely measured in the fasting state, excluding CM and their remnants; however, elevated non-fasting TG levels were found to be associated with an increased risk of coronary artery disease, stroke and death in men and women.\textsuperscript{6-8} which suggests atherosclerosis as a postprandial phenomenon where CM and CM remnants would play an important role. Thus, increased levels of non-fasting TG, as well as increased levels of CM and CM remnants, should constitute a potentially important predictor of atherosclerotic cardiovascular diseases, and the strong evidence supporting the independent atherogenicity of these remnants\textsuperscript{9} makes them appropriate targets for lipid-lowering therapy.

CD36, also known as fatty acid (FA) translocase, an 88 kD glycoprotein belonging to the scavenger receptor class B, has been shown to bind multiple ligands, including long-chain FAs and oxidized low density lipoproteins.\textsuperscript{10} CD36 is broadly expressed in many cells, such as monocytes, platelets, macrophages, microvascular endothelial cells, adipocytes, skeletal and cardiac myocytes, enterocytes and Kupffer cells.\textsuperscript{11} Human CD36 deficiency is accompanied by multiple risk factors, such as increased remnant lipoproteins and low high density lipoproteins (HDL) cholesterol, as well as impaired glucose metabolism, based upon insulin resistance. These findings suggested that this condition may be considered a genetic background for MetS.\textsuperscript{12, 13} CD36 knockout (CD36KO) mice have been also demonstrated to increase the postprandial plasma TG and FA response after an acute oral fat loading of more than 2-fold compared to wild-type (WT) mice.\textsuperscript{14} We demonstrated a postprandial increase of plasma CM-remnants with enhanced TG synthesis in the small intestine of CD36KO compared to WT mice and suggested that the main cause for the postprandial elevation of TG in plasma was the de novo synthesis of small-sized CM in enterocytes.\textsuperscript{15} These findings strongly suggest CD36KO mice as an interesting model to evaluate postprandial hypertriglyceridemia in a MetS environment.

Peroxisome proliferator activated receptor (PPAR) alpha is a ligand-activated transcription factor with diverse functions, expressed in a variety of tissues,\textsuperscript{16} and is activated by several synthetic compounds. Fenofibrate, a PPAR-\alpha ligand, has been demonstrated to reduce TG levels in fasting and postprandial states in a cohort of hypertriglyceridemic subjects with MetS;\textsuperscript{17} this TG-lowering effect resulted primarily from reductions in fasting and postprandial concentrations of large and medium VLDL particles.\textsuperscript{17} Moreover, fenofibrate has been shown to reduce non-fatal myocardial infarctions and coronary revascularizations in diabetic patients.\textsuperscript{18}

To elucidate the effect of fenofibrate on postprandial hypertriglyceridemia in CD36KO mice, we performed an oral fat-loading test before and after fenofibrate treatment and demonstrated that fenofibrate reduced postprandial hypertriglyceridemia, thus promoting a protective effect against atherosclerosis in a mouse model for MetS.

**Materials and Methods**

**Animals**

Male CD36KO mice created on a C57BL6/J background, which were kindly provided by Mason. W. Freeman, M.D., Ph.D., Professor of Harvard Medical School,\textsuperscript{19} and C57BL6/J WT mice at 8–10 weeks of age were used for this experiment. Each strain of mice was separated into two groups, which were fed chow diet (MF, Oriental BioLaboratories, Chiba, Japan) alone or chow diet containing 0.05% fenofibrate (Aska Pharmaceuticals, Tokyo, Japan) for 2 weeks. The mice were housed in a temperature-controlled environment with a 12-hour dark-light cycle and free access to food and water. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine (IEXAS). After 2 weeks of treatment, each strain was fasted for 12 hours and separated into two groups to be euthanized: in the fasting state and three hours after acute ingestion of 17 \( \mu \)L/g body weight of olive oil (Nacalai Tesque, Kyoto, Japan) by gavaging.

**Triglyceride Determination and Lipoprotein Analysis of Plasma and Intestinal Lymph**

Plasma and lymph TG concentrations were measured enzymatically (Wako Pure Chemical Industries, Tokyo, Japan) according to the manufacturer’s protocol.

The plasma and lymph lipoprotein profile was analyzed by an online dual enzymatic method using high performance liquid chromatography (HPLC) at Skylight Biotech Inc. (Akita, Japan), where 200 \( \mu \)L of plasma or lymph were dissolved in loading buffer and loaded onto TSK gel Lipropopak XL columns. Triglyceride concentrations in the flow-through were measured continuously and simultaneously. The corresponding lipoprotein fractions (CM, VLDL, LDL, and HDL-sized fractions) and the elution time were CM (>80 nm, fraction time: 15–17 min), VLDL (30–80 nm, fraction time: 17–22 min), LDL (16–30 nm, fraction time: 22–28 min), and HDL (8–16 nm,
fraction time: 28–37 min).

Collection of Intestinal Lymph in the Postprandial State

Five mice from each strain were loaded with olive oil (17 μL/g body weight) after a fasting period of 12 hours. Three hours later, mice were anesthetized and the intestinal lymphatic trunk was cannulated using a 27-gauge needle inserted into a polyethylene tube (PE-50) previously flushed with EDTA-treated water, according to the modified method described by Bollman et al.21).

Western Blot

One microliter of plasma or lymph was subjected to 4–12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE; TEFCO, Tokyo, Japan), transferred onto an Immobilon-P transfer membrane (Millipore Corp., USA) and blocked by Blocking One (Nacalai Tesque, Kyoto, Japan). The membrane was then incubated with anti-mouse apoB-48/B-100 antibody (BIODESIGN International, ME, USA) and anti-rabbit IgG antibody (NA934V; GE Healthcare Backinghamshire, UK). Bands corresponding to apoB-100 and apoB-48 were detected with the ECL Advance Detection Kit (GE Healthcare, UK).

RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR

The small intestine from each animal was removed, flushed with ice-cold phosphate-buffered saline and divided into three sections of equal length, the proximal two-thirds of the mucosa was gently scraped and stored in RNAlater RNA stabilization reagent (QIAGEN GmbH, Germany) at −20°C. Total RNA from tissue samples was extracted and purified using the RNaseasy Lipid Tissue Mini Kit (cat. 70804; QIAGEN GmbH, Germany). One microgram of the total RNA was primed with 50 pmol of oligo (dT) 20 and transcribed with SuperScript III (Invitrogen, CA, USA) for first-strand cDNA synthesis, according to the manufacturer’s protocol. qRT-PCR was performed; DNA polymerase and SYBR Green I (Finnzymes Oy, Espoo, Finland) were set in a reaction volume of 20 μL containing gene-specific primers (5 μM) and cDNA (corresponding to ~50 ng total RNA). The reaction was performed using the DNA engine Opticon 2 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The \(2^{-\Delta\Delta CT}\) method of relative gene expression was employed and a standard deviation of ct value of <0.3 was accepted. Results are expressed as arbitrary units in comparison with the expression of GAPDH.

Primers for this Study

The sequence data of the genes were found in GenBank and the sequences of primers were designed with Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). GAPDH was used as a housekeeping gene. The sequence and information for primers used in this study are as follows: FATP-4 (GenBank accession number NM_011989): 5′-atcaacaccaacctt-aggcc-3′ and 5′-aacccttctgtcggagtctg-3′, FABP1 (NM_017399): 5′-catcaagagggaggaagca-3′ and 5′-tttccccagtctaggcccc-3′, FABP2 (NM_007980): 5′-tctggtctcgag-aggttct-3′ and 5′-gccttgagcctggtcagc-3′, DGAT-1 (NM_010046): 5′-gtgacagatgccagct-3′ and 5′-cggatgctgactgcatc-3′, and DGAT-2 (NM_026384): 5′-aggctgcaacctctatcagct-3′ and 5′-aaggaaataggtggagcagata-3′, MGAT-2 (NM_177448): 5′-gaagaacaagcagcgac-3′ and 5′-tggagttctagggcag-3′, ApoB (NM_009693): 5′-tgtggacctctcctgtctgag-3′ and 5′-gtgagcgatcagctaatggttg-3′, MTTP (NM_008642): 5′-ctgctctgagtcggcttgcct-3′ and 5′-ctctgtgagccagactgta-3′, and GAPDH (NM_008084): 5′-actccactcagcgaaatatc-3′ and 5′-tctctcttggtgaggagca-3′.

Statistical Analysis

The values are expressed as the means ± S.D. Statistical significance was assessed by Student’s \(t\)-test for paired values and set at \(p<0.05\).

Results

Fenofibrate Reduces Postprandial Hypertriglyceridemia, as well as ApoB-100 and ApoB-48 Mass in Wild-Type and CD36KO Mice in Fasting and Postprandial States

CD36KO mice showed significantly higher plasma TG levels than WT controls (638 ± 123 mg/dL vs. 168 ± 27 mg/dL, \(p<0.05\)) in the postprandial state (Fig. 1). Administration of fenofibrate decreased plasma TG concentrations in the fasting state in both WT (87 ± 32 vs. 21 ± 2 mg/dL, \(p<0.05\)) and CD36KO mice (82 ± 11 vs. 23 ± 4 mg/dL, \(p<0.05\)). Moreover, fenofibrate markedly reduced the postprandial plasma TG concentration in CD36KO mice (638 ± 123 vs. 45 ± 20 mg/dL, \(p<0.05\)), while the reduction of TG in WT mice was somewhat modest compared to that in CD36KO mice (168 ± 27 vs. 52 ± 14 mg/dL, \(p<0.05\)). This marked diminution of the TG level in the postprandial state in CD36KO mice after fenofibrate treatment implies that fenofibrate could act more efficiently in the postprandial state in the MetS environment. It is important to point out that fenofibrate administration did not affect mouse weight significantly during the 2-week treatment in both CD36KO
and WT groups compared to their chow diet controls (data not shown).

To assess the effect of fenofibrate administration on apoB-48 mass in the plasma of WT and CD36KO mice in fasting and postprandial states, Western blotting was performed. The amount of both apolipoprotein B isoforms, apoB-100 and apoB-48, in plasma was markedly reduced after fenofibrate treatment in both states and strains (Fig. 1), implying that both apoB-100- and apoB-48-containing lipoproteins were reduced.

**Fenofibrate Reduces Postprandial CM and VLDL-Sized Particles in Plasma of CD36KO Mice**

The plasma lipoprotein profile was analyzed by automatic HPLC using a pool of 3 samples for each group. CD36KO mice showed a marked increase in postprandial TG levels of every lipoprotein fraction compared to their WT controls before fenofibrate administration. Among subfractions, a substantial difference between CD36KO and WT mice was demonstrated in TG levels of CM and VLDL-sized particles, which also include CM remnants, indicating that CD36KO mice showed impaired TG-rich lipoprotein metabolism in the postprandial state (Fig. 2A). Fenofibrate reduced postprandial TG levels in WT and CD36KO mice mainly in these subfractions (Fig. 2B, 2C). Fig. 2D shows the overall HPLC analysis of CD36KO mouse plasma in the postprandial state before and after fenofibrate treatment. These results raised the possibility that fenofibrate could modulate intestinal CM production. Thus, we further investigated the lipoproteins in the intestinal lymph and intestinal mRNA expression of genes in CD36KO mice in the postprandial state before and after fenofibrate treatment.

**Fenofibrate Reduces Postprandial TG and ApoB-48 Mass in Intestinal Lymph of CD36KO Mice**

Fenofibrate significantly reduced the postprandial TG concentration in the intestinal lymph of CD36KO mice in the postprandial state (18.6 ± 2.2 vs. 10.0 ± 1.6 g/dL, p < 0.05) accompanied by a decrease in apoB-48 mass (Fig. 3A). The highest peak in TG levels corresponded to the CM fraction in both treated and non-treated mice, with a discrete elevation in the VLDL-sized fraction, which corresponds to CM remnants, since the obtained lymph lacked apoB-100 (Fig. 3B). Fenofibrate decreased both CM and CM remnant-sized curves, suggesting that fenofibrate might decrease the production of intestine-derived lipoproteins in the postprandial state in CD36KO mice (Fig. 3B).
Fenofibrate is Involved in the Transcriptional Regulation of Lipid Metabolism-Related Genes in Intestine of CD36KO Mice in Postprandial State

To determine the possible mechanisms involved in the attenuation of postprandial hypertriglyceridemia by fenofibrate, qRT-PCR using isolated total intestinal mRNA was performed and the expression of genes associated with FA and TG transport as well as...
CM assembly in the intestine of CD36KO mice treated and non-treated with fenofibrate was examined. First we investigated the intestinal PPARalfa expression to confirm the efficacy of fenofibrate treatment in this experiment. Fenofibrate administrated for two weeks to CD36KO mice increased the intestinal mRNA expression of PPARalfa 2-fold.

The mRNA expression of diacyl glycerol acyl transferase (DGAT)-1, DGAT-2, and monoacyl glicerol acyl transferase (MGAT)-2, which are involved in the intracellular formation of TG in intestinal epithelial cells, did not change significantly (Fig. 4).

ApoB mRNA was found to be decreased in mice fed with fenofibrate, while the genes that participate in apoB mRNA production, apobec-1 and apobec-1 complementation factor (ACF), were not affected significantly, which suggests the decrease of intestinal apoB mRNA as a determinant factor in the inhibitory action of fenofibrate on CM production (Fig. 4).

**Fig. 3.** Fenofibrate reduces postprandial TG and apoB-48 mass in intestinal lymph of CD36KO mice.

(A) Fenofibrate treatment (black bar) significantly reduced postprandial TG in intestinal lymph of CD36KO mice, and also notably decreased the apoB-48 mass 3 hours after the ingestion of a fat load. (B) HPLC curves of lymphatic lipoproteins in postprandial state before (solid black line) and after (solid gray line) fenofibrate treatment in CD36KO mice.
Interestingly, microsome triglyceride transfer protein (MTP) mRNA expression, considered to have an important role in CM assembly in epithelial cells, was not significantly altered by the presence of fenofibrate.

Discussion

The TG-lowering effect of fenofibrate has been widely reported to occur mainly via the activation of lipoprotein lipase (LPL) by increased hepatic LPL mRNA levels and by suppression of liver mRNA levels of apoCIII, which is a potent inhibitor of LPL. The former was supported by the finding of a peroxisome proliferator-response element (PPRE) in the human LPL gene.22 Fenofibrate also down-regulates lipogenic genes in the liver, such as fatty acid synthase, acetyl CoA carboxylase, and DGAT-2, inducing hepatic FA uptake and reducing FA synthesis and VLDL production in hepatocytes, thereby directly affecting the catabolism of TG-rich lipoprotein.23, 24

As described above, the mechanism of action of fenofibrate in the TG-lowering effect was largely centered on the liver and could explain in part the marked reduction of VLDL-sized CM remnants observed in the plasma of treated CD36KO mice (Fig. 2D). However, little is known about the effect of fenofibrate on TG metabolism in the intestine. We did not determine LPL activity in our study, already mentioned as a crucial factor in the TG-lowering effect of fenofibrate, since we focused on the mechanisms concerning the intestinal production of ApoB-containing lipoproteins. This study added a novel mechanism of the TG-lowering effect of fibrates, that is, the production of intestine-derived lipoproteins, CM and CM remnant-sized particles, was inhibited by fenofibrate (Fig. 3B).

It is known that CD36KO mice present an increased hypertriglyceridemic response to both oral fat loading and chronic exposure to a high fat diet compared to WT mice.14 Our laboratory previously found an increased TG concentration and apoB-48 mass in the intestinal lymph of CD36KO mice in fasting and postprandial states, without any alteration in lipoprotein lipase (LPL) or hepatic lipase activity between CD36KO and WT mice, highly suggesting that the postprandial hypertriglyceridemia observed in this animal model is due to increased CM production from the intestine.15 In the present study, we demonstrated that the PPAR-α agonist fenofibrate was able to decrease postprandial TG levels in plasma and intestinal lymph of CD36KO mice.

Our results also showed a statistically significant reduction in the postprandial apoB mRNA expression of CD36KO mice treated with fenofibrate, which might suggest this as the mechanism responsible for reduced CM production. However, the regulation of apoB has been largely reported to be posttranscriptional, although it is also true that most of these studies were not performed in intestinal cells but in hepa-
tocytes. Fu et al. reported the PPAR-α agonist ciprofibrate as an inhibitor of the expression of ACF, one of the responsible factors of apoB mRNA production; however, this inhibition was found only in the liver, not in the intestine of LDL-receptor knockout mice. This supports the idea that the factors involved in the regulation of apoB lipoproteins, including PPAR-α agonists, might differ between these two tissues, which leads to the need for more studies to understand the regulation of apoB in the small intestine.

MTP catalyzes the transfer of TG and cholesteryl esters to apoB and therefore has a main role in the assembly of apoB-containing lipoproteins. It has been reported that PPAR-α agonists increase MTP expression and apoB secretion in rodent liver but not in the intestine in spite of decreased plasma TG levels. We found that MTP expression was not affected by fenofibrate in the intestine of CD36KO mice in the postprandial state, which also contributes to the idea that regulation of the production of apoB-containing lipoproteins in the intestine might be different from the liver.

Our results show fenofibrate to be an effective treatment for postprandial hypertriglyceridemia in CD36KO mice, and the reduction in the intestinal production of ApoB-containing lipoproteins as a new mechanism of action for this drug. Thus, since human CD36 deficiency is a genetic background of metabolic syndrome, as stated previously, we suggest that fenofibrate might play a similar role not only in CD36-deficient patients, but in MetS; this hypothesis, however, needs to be tested in further studies.

**Conclusion**

Fenofibrate reduces postprandial hypertriglyceridemia in CD36 knockout mice; this reduction is associated with the inhibition of intestinal apoB-48 production and the subsequent reduction of intestinal apoB-containing lipoproteins. This suggests a protective effect of fenofibrate against atherosclerosis in CD36KO mice as a monogenic model of metabolic syndrome.

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