does not accelerate the degradation of nuclear SREBP-1 protein (11).

We have shown that two mechanisms are involved in the PUFA regulation on SREBP-1; one is at the proteolytic processing level, and the other is at the transcription level. What is the physiological role of this two-step regulation? As we have shown in Fig. 1, the proteolytic mechanism is high sensitive, although the transcriptional mechanism is low sensitive, consistent with the previous report by Ezaki and co-workers (35). The combination of these two steps of regulation with different sensitivity connected in series probably helps to achieve the broader responsive range of the amount of PUFA.

One of our conclusions is that LXR is not involved in the transcriptional regulation by PUFA, but controversy exists over this point; several previous reports suggested the involvement of LXR in this regulation (15, 16), whereas others did not (36). These previous studies have been all performed in *in vitro* settings, and therefore we evaluated the contribution of the LXR pathway in the *in vivo* setting for the first time, and we have concluded that the involvement of LXR is not detectable, although LXR is an important determinant of the SREBP-1 expression level.

Because an *in vitro* model is always only a small part of the whole *in vivo* system, how large or small the contribution of a regulatory pathway elucidated *in vitro* is in the whole *in vivo* system cannot be estimated until it is assessed in the in *vivo* setting. To address these issues, our approach of the extension of *in vitro* reporter assays to *in vivo* settings will be very useful in various situations.

We have demonstrated that PUFA selectively suppresses the proteolytic processing of SREBP-1, but the molecular mechanism underlying this SREBP-1-specific regulation is currently unknown. SCAP escorts both SREBP-1 and -2 to the Golgi, and SREBP-1-specific adaptor protein has not been reported yet. Recently, an endoplasmic reticulum membrane protein TRC8 (translocation in renal cancer from chromosome 8) has been documented to hamper endoplasmic reticulum-to-Golgi transport of SREBP-2/SCAP and reduce SREBP-2 cleavage specifically (37). Perhaps there might be some adaptor molecule that specifically interacts with SREBP-1 and mediates the suppressive effect of PUFA, although we have no evidence. If the molecular mechanism underlying this SREBP-1-specific effect of PUFA is clarified in the future, it will be a potential molecular target for new lipid-lowering drugs. In conclusion, the primary mechanism for PUFA suppression of SREBP-1 expression is at the proteolytic processing level and that this suppression in turn decreases the transcription of *Srebf1c* through lowering SREBP-1 binding to SRE on the promoter.

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Original Article

Molecular Mechanisms of Ezetimibe-Induced Attenuation of Postprandial Hypertriglyceridemia

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Aim: Postprandial hypertriglyceridemia (PHTG) has been shown repeatedly to be associated with metabolic syndrome and atherosclerotic cardiovascular diseases. We have recently reported that ezetimibe inhibits PHTG in patients with type IIb hyperlipidemia. Ezetimibe was also reported to attenuate PHTG in combination with low-dose statins in patients with obesity or metabolic syndrome. We reported CD36-deficient (CD36KO) mice as a new model for PHTG, in which the synthesis of chylomicron (CM) in the small intestines is enhanced. In the current study, we investigated the effect of ezetimibe on PHTG in this mouse model of metabolic syndrome.

Methods: Wild-type (WT) mice fed a western diet, and CD36KO mice fed a normal chow diet, respectively, were treated for 3 weeks with and without ezetimibe, followed by an evaluation of triglyceride (TG) concentrations by enzymatic method and by high performance liquid chromatography (HPLC) as well as those of and apolipoprotein (Apo) B-48 in plasma and intestinal lymph after oral fat loading with olive oil. Intestinal mucosa was also harvested to evaluate the transcriptional regulation of the genes involved in the intestinal production of ApoB-containing lipoproteins.

Results: Ezetimibe dramatically reduced PHTG in both WT and CD36KO mice. HPLC analysis of plasma showed that the decrease in TG content in CM and CM remnants-sized particles contributed to this suppression, suggesting that CM production in the small intestines might be reduced after ezetimibe treatment. Intestinal lymph was collected after oral fat loading in ezetimibe-treated and non-treated mice. Both TG content and ApoB-48 mass were decreased in ezetimibe-treated mice. The quantitative RT-PCR of intestinal mucosa showed down-regulation of the mRNA expression of FATP4 and ApoB in both groups along with FABP2, DGAT1, DGAT2 and SCD1 in WT mice at postprandial state after ezetimibe treatment.

Conclusion: Ezetimibe alone reduces PHTG by blocking both the absorption of cholesterol and the intracellular trafficking and metabolism of long-chain fatty acids in enterocytes, resulting in the reduction of the formation of ApoB-48 which is necessary for the ApoB48-containing lipoprotein production in the small intestines.

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Key words; Postprandial hypertriglyceridemia, Ezetimibe, CD36 deficiency, Long-chain fatty acids, Apolipoprotein B-48

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Introduction

Metabolic syndrome (MetS) is defined as a cluster of interrelated factors commonly associated with atherosclerotic cardiovascular diseases: central obesity, modestly high blood pressure, impaired glucose

metabolism and atherogenic dyslipidemia[1], including elevated triglyceride (TG) in the fasting state.

Besides the early hours of the day before breakfast, we are constantly in a non-fasting state. Accumulating evidence concerning nonfasting TG levels as a predictor of cardiovascular diseases[2] and stroke[3] suggest atherosclerosis as a postprandial phenomenon in which intestine-derived TG-rich lipoproteins, such as chylomicron (CM) and CM remnants, would play an important role[4-6], which Zilversmit stated three decades ago[7].

CD36, or fatty acid translocase, is an 88 kD scavenger receptor class B that is expressed in many cells, such as monocytes, macrophages, microvascular endothelial cells, adipocytes, skeletal and cardiac myocytes and enterocytes. It binds multiple ligands, including long-chain fatty acids (FAs) and oxidized low density lipoprotein[8]. Patients with CD36 deficiency present with increased remnant lipoproteins and decreased high density lipoprotein (HDL)-cholesterol, as well as impaired glucose metabolism based upon insulin resistance. All these findings suggest that CD36 deficiency may be considered a monogenic form of MetS[9]. CD36 knockout (CD36KO) mice present with an excessive postprandial plasma TG and FA response after acute oral fat loading compared to wild-type (WT) mice[10]. Previous studies in our laboratory using CD36KO mice reported a postprandial increase in plasma CM and CM remnants with enhanced TG synthesis in the small intestines, suggesting that the main cause of postprandial hypertriglyceridemia (PHTG) in CD36KO mice was the increased *de novo* synthesis of small CM in enterocytes[11]. These findings established CD36KO mice as a model to evaluate PHTG in a MetS environment.

Ezetimibe, a cholesterol absorption inhibitor that acts by blocking the sterol-induced internalization of the key cholesterol transporter, Niemann-Pick C1 Like 1 (NPC1L1), in enterocytes[12] has been demonstrated to lower total and LDL-cholesterol levels significantly in patients with primary and mixed hypercholesterolemia as a coadjuvant therapy to either statins[13] or fibrates[14]. In these studies, ezetimibe was also found to decrease other important atherogenic factors significantly, such as fasting TG and total apolipoprotein B (ApoB) levels in plasma. Moreover, ezetimibe has been demonstrated to reduce PHTG in combination with low-dose statins in patients with obesity and metabolic syndrome comparable to high-dose statins alone[15].

Recently, our group reported that ezetimibe alone significantly reduced PHTG in Japanese subjects with type IIb hyperlipidemia[16], suggesting that ezetimibe might also play a role in regulating the production of TG-rich lipoproteins in addition to act as a cholesterol absorption inhibitor. Since investigations concerning ezetimibe and its mechanism of action on lipid metabolism have primarily focused on sterol metabolism, we prioritized the need to establish molecular mechanisms that participate in the TG-lowering effect of ezetimibe in the postprandial state. For that purpose, we performed oral fat loading in ezetimibe-treated and non-treated wild-type (WT) mice fed a western diet and CD36KO mice fed a chow diet as an animal model of PHTG. We demonstrated that ezetimibe reduces PHTG by decreasing the absorption of both cholesterol and long-chain FAs through enterocytes, which affected intestinal FA transport, TG production, and CM formation in both mice strains.

Materials and Methods

Animals

Male C57BL6/J WT and CD36KO mice created on a C57BL6/J background (kindly provided by Mason. W. Freeman, M.D., Ph.D., Professor of Harvard Medical School)[17], 8–10 weeks of age were used for this experiment. Each mouse strain was separated into two groups in the following manner: CD36KO mice were fed a chow diet (MF; Oriental BioLaboratories, Chiba, Japan) either with or without supplementation of 10 mg/kg ezetimibe (Schering-Plough, USA), and WT mice were fed a western diet either with or without supplementation of 10 mg/kg ezetimibe. The animals were housed in a temperature-controlled environment at 12-hour dark-light cycles with free access to food and water. After 3 weeks of treatment, mice in each group were divided into 2 subgroups. One subgroup was euthanized after fasting for 12 h and the other was fasted for 12 h followed by acute ingestion of 17 μL/g body weight of olive oil (Nacalai Tesque, Kyoto, Japan) by intragastric gavage, and then euthanized 3 h after initiating oral fat loading. Plasma, intestinal lymph and tissues were collected from both subgroups at the time of euthanization. Additionally, WT mice fed a standard chow diet were used as controls for the TG determination study. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University Graduate School of Medicine (IEXAS).

Lipid Determination and Lipoprotein Analysis of Plasma and Intestinal Lymph

Cholesterol and TG concentrations in plasma and intestinal lymph for each mouse were measured

using an enzymatic method (Wako Pure Chemical Industries, Tokyo, Japan) according to the manufacturer's protocol. Plasma and lymph lipoprotein profiles were analyzed by an online dual enzymatic method using high performance liquid chromatography (HPLC) at Skylight Biotech Inc. (Akita, Japan)[18]. Two hundred microliters of plasma or lymph were dissolved in loading buffer and loaded onto TSK gel Lipopropak XL columns; TG concentrations in the flow-through were measured continuously and simultaneously. The correspondence of the size of lipoprotein fractions (CM, very low density lipoprotein (VLDL), LDL, and HDL-sized fractions) and the elution time were; CM (particle diameter >80 nm, elution time: 15–17 min), VLDL (particle diameter: 30–80 nm, elution time: 17–22 min), LDL (particle diameter: 16–30 nm, elution time: 22–28 min), and HDL (particle diameter: 8–16 nm, elution time: 28–37 min).

Collection of Intestinal Lymph in Postprandial State

Five mice from each group, previously fasted for 12 h, were gavaged with olive oil (17 μL/g body weight). Three hours later, the animals were anesthetized and the intestinal lymphatic trunk was cannulated with a 27-gauge needle connected to a polyethylene tube (PE-50), which was pretreated with EDTA-containing water. The procedure was performed in accordance with the modified method described by Bollman *et al.*[19]. The collected intestinal lymph was used for HPLC and protein detection by western blot.

Determination of Labeled Triolein Absorption

Mice from each group were fasted for 12 h and gavaged with 3 μCi of [9,10-^{3}H(N)] triolein (PerkinElmer, MA, USA) mixed into 17 μL/g body weight of olive oil. Three hours after fat loading, the mice were euthanized and blood samples were collected from the inferior vena cava. The activity of radiolabeled tritium in 250 μL plasma was determined by scintillation counting using a WALLAC Winspectral™ 1414 Liquid Scintillation Counter.

Protein Detection by Western Blot

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

RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR

Mice were fasted for 12 h, gavaged with olive oil as previously stated, and their small intestines were removed, flushed with ice-cold phosphate-buffered saline and divided into three sections of equal length; the proximal two-thirds of mucosa were gently scraped and stored in RNAlater RNA stabilization reagent (QIAGEN GmbH, Germany) at −20℃.

Total RNA from tissue samples were extracted and purified using the RNeasy Plus Mini Kit (cat. 74134; QIAGEN GmbH, Germany). Two micrograms of total RNA were primed with 50 pmol anchored-oligo $(dT)_{18}$ and transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany), according to the manufacturer's protocol. Quantitative RT-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 standard deviation with a ct value of <0.3 was accepted. Results are expressed as arbitrary units in comparison with the expression of GAPDH.

Primers Used for This Study

The sequence data of the genes were found with 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: CD36 (GenBank accession number NM_001159558): 5'-gagcaactggtggatggttt-3' and 5'-gcagaatcaagggagagcac-3', FATP-4 (NM_011989): 5'-atcaacaccaaccttaggcg-3' and 5'-aacccttgtctgggtgactg-3', FABP1 (NM_017399): 5'-catccagaaagggaaggaca-3' and 5'-ttttccccagtcatggtctc-3', FABP2 (NM_007980): 5'-ttgctgtccgagaggtttct-3' and 5'-gctttgacaaggctggagac-3', FAS (NM_007988): 5'-gctgcggaaacttcaggaaat-3' and 5'-agagacgtgtcactcctggactt-3', SCD1 (NM_009127): 5'-ccttccccttcgactactctg-3' and 5'-gccatgcagtcgatgaagaa-3', DGAT-1 (NM_010046): 5'-gtgcacaagtggtgcatcag-3' and 5'-cagtgggatctgagccatc-3', DGAT-2 (NM_026384): 5'-agtggcaatgctatcatcatcgt-3' and 5'-aaggaataagtgggaaccagatca-3', MGAT-2 (NM_

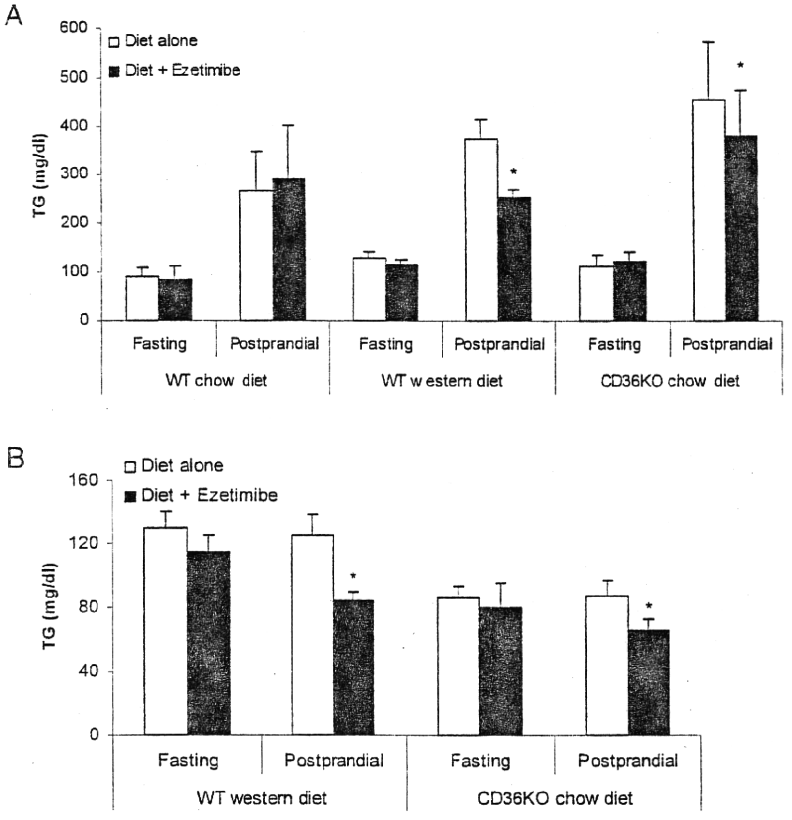

Fig. 1. Ezetimibe Reduces Postprandial Hypertriglyceridemia in Both CD36KO and WT Mice.

CD36KO mice fed a standard chow diet and WT mice fed a western diet, respectively, showed significantly higher plasma TG levels than WT fed a standard chow diet in the postprandial state (white bars) in non-treated groups. Administration of ezetimibe (black bars) decreased plasma TG concentrations at postprandial state in both CD36KO and WT fed a western diet but not in WT mice fed a standard chow diet (A). Ezetimibe also reduced the postprandial concentration of total cholesterol in plasma of both study groups (B). ($^*p < 0.05$)

177448): 5'-gaagaagcagcatcagggac-3' and 5'-gtgtgggatt-agggggactt-3', ApoB (NM_009693): 5'-tgggattccatct-gccatctcgag-3' and 5'-gtagagatccatcacaggacaatg-3', Apobec1 (NM_031159): 5'-accacaacggatcagcgaaa-3' and 5'-tcatgatctggatagtcacaccg-3', ACF (NM_001081074): 5'-agccagaatcctgcaatcc-3' and 5'-agcata-cctcttcgcttcatcc-3', ACSL1(NM_007981): 5'-tgacctc-tccatgcagtcag-3' and 5'-agcctatgcactcagcgagt-3', HMGCR (NM_008255): 5'-ctggaattatgagtgccccaaa-3' and 5'-acgactgtactgaagacaaagc-3', ACAT2 (NM_009338): 5'-tgtcacagaacagggcagag-3' and 5'-tgacagttcc-tgtcccatca-3' MTTP (NM_008642): 5'-catgtcagccatcct-gtttg-3' and 5'-ctcgcgataccacagactga-3', and GAPDH (NM_008084): 5'-actccactcacggcaaattc-3' and 5'-tctc-catggtggtgaagaca-3'.

Statistical Analysis

The values were 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

Ezetimibe Reduces Postprandial Hypertriglyceridemia in Both CD36KO and Wild Type Mice

CD36KO mice fed a standard chow diet and WT mice fed a western diet showed significantly higher plasma TG levels than WT fed a standard chow diet in the postprandial state without ezetimibe treatment (CD36KO 457 ± 114 mg/dL, WT western diet 376 ± 41 mg/dL vs WT chow diet 267 ± 81 mg/dL. $n = 25$). Administration of ezetimibe decreased plasma

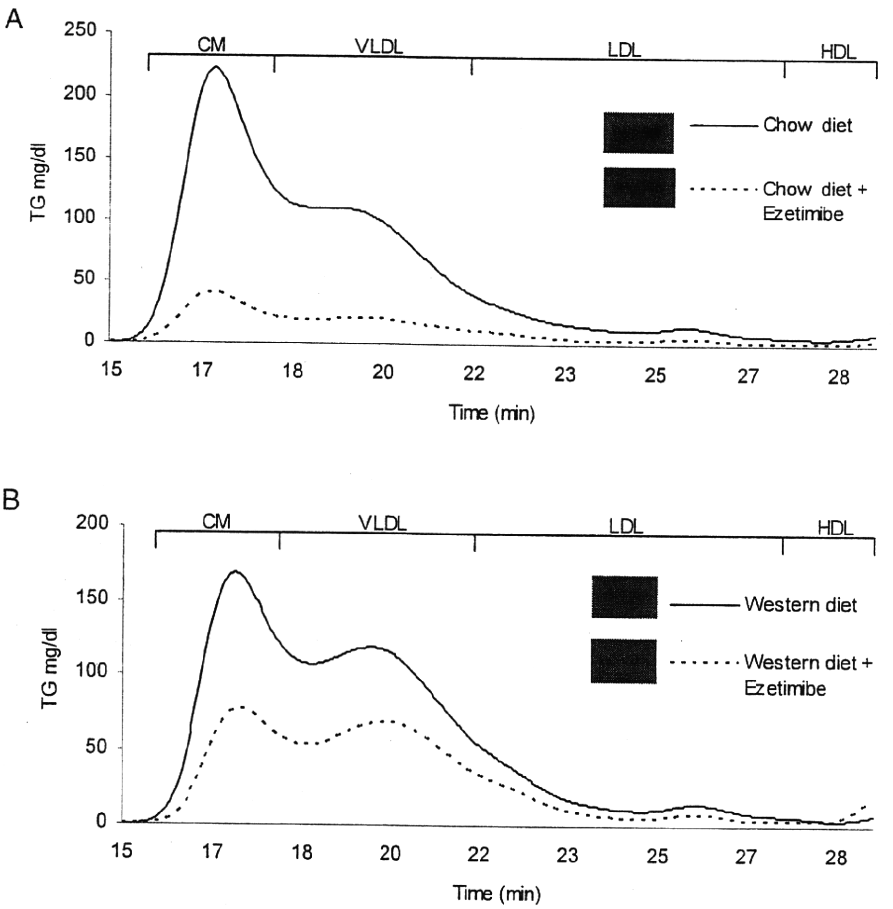

Fig. 2. Ezetimibe Reduces Postprandial CM- and VLDL-sized Particles As Well As ApoB48 Mass in Plasma of WT and CD36KO Mice.

Plasma lipoprotein profile was analyzed by HPLC. Ezetimibe (dotted line) reduced the average postprandial TG levels in both CD36KO (A) and WT mice (B) in CM- and VLDL-sized subfractions, which corresponded to CM remnants. Moreover, ezetimibe decreased the ApoB48 mass in plasma in both groups (representative sample). These results support the idea that ezetimibe might have some modulating effect on intestinal CM production.

TG concentrations in the postprandial state in both CD36KO and WT fed a western diet but not in WT mice fed a standard chow diet (**Fig. 1A**) as well as plasma total cholesterol concentrations in plasma in both study groups (**Fig. 1B**). The selective decrease in both postprandial TG levels suggests that the ezetimibe action on plasma TG concentrations is enhanced by a postprandial MetS environment, since both affected groups are indeed animal models of postprandial hyperlipidemia.

Ezetimibe Reduces Postprandial CM and VLDL-Sized Particles as Well as ApoB48 Mass in Plasma of WT and CD36KO Mice

Plasma lipoprotein profile was analyzed by HPLC using five samples for each group. The highest peak corresponded to CM- and VLDL-sized fractions in both ezetimibe-treated and non-treated mice in both groups. We found that ezetimibe reduced postprandial TG levels in both WT and CD36KO mice mainly in CM- and VLDL-sized subfractions, which corresponded to CM remnants (**Fig. 2A** and **2B**). Moreover, ezetimibe decreased the ApoB48 mass in plasma in both groups. These results support the idea that ezetimibe might have some modulating effect on intestinal CM production. Thus, we further investigated lipoproteins in the intestinal lymph, the intestinal absorption of tritium-labeled FAs, and intestinal mRNA expression of a variety of genes involved in CM synthesis in both strains of mice in the postpran-

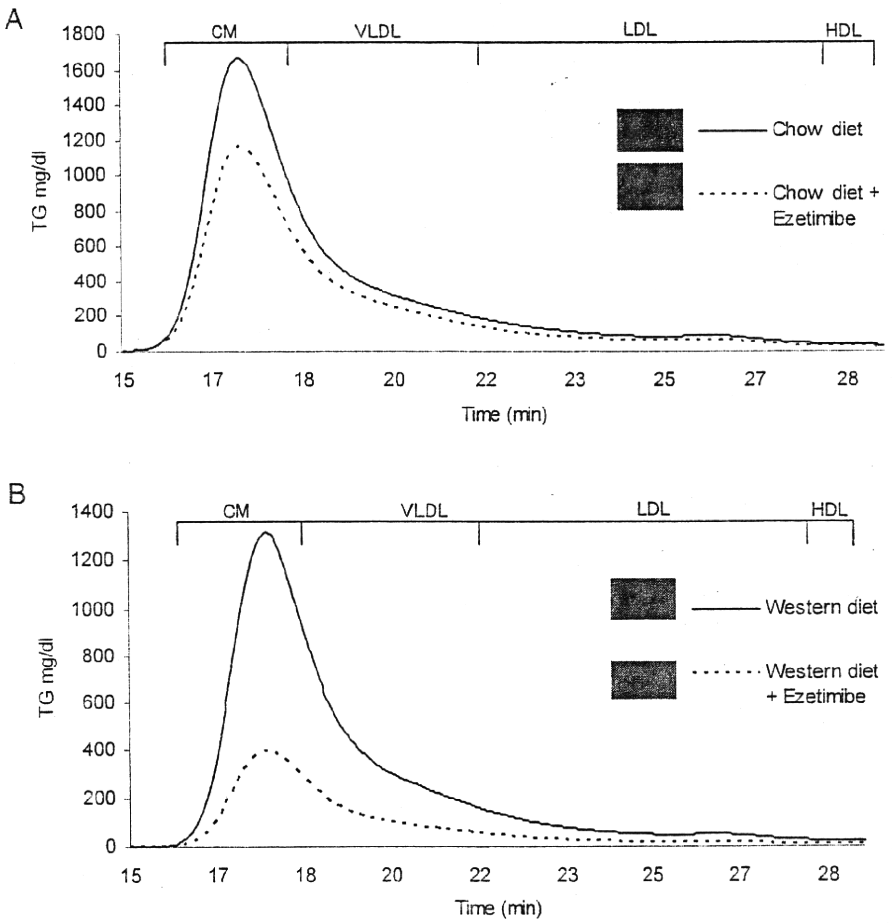

Fig. 3. Ezetimibe Reduces Postprandial TG and ApoB48 Mass in Intestinal Lymph of WT and CD36KO Mice.

HPLC analysis showed that ezetimibe (dotted lines) reduced significantly the average postprandial TG concentration in the intestinal lymph of CD36KO (A) and WT (B) mice in the postprandial state; this reduction was accompanied by a decrease in ApoB48 mass. Ezetimibe decreased the CM peak in both groups, suggesting that it might act by lowering the production of intestine-derived lipoproteins in the postprandial state.

dial state with and without ezetimibe treatment.

Ezetimibe Reduces Postprandial TG and ApoB48 Mass in Intestinal Lymph of WT and CD36KO Mice

Ezetimibe reduced the postprandial TG concentration significantly in intestinal lymph of both study groups in the postprandial state; this reduction was accompanied by a decrease in apoB48 mass in lymph. The highest peak in TG levels corresponded to the CM fraction in treated and non-treated mice in both groups. Ezetimibe decreased the CM peak, suggesting that it might act by lowering the production of intestine-derived lipoproteins in the postprandial state in both groups of mice (**Fig. 3A** and **3B**).

Ezetimibe Reduces the Intestinal Absorption of Radio-Labeled Triolein

To investigate the possible mechanisms by which ezetimibe reduced the intestinal TG secretion, we evaluated intestinal FA absorption. Ezetimibe-treated and non-treated mice from both strains were loaded with 17 μL/g olive oil containing 3 μCi of [9,10-^{3}H(N)] triolein. At 3 h after oral fat loading, ezetimibe significantly reduced ^{3}H radioactivity in the plasma of both strains (**Fig. 4A** and **4B**), establishing that there is a reduction in intestinal FA absorption associated with the administration of ezetimibe.

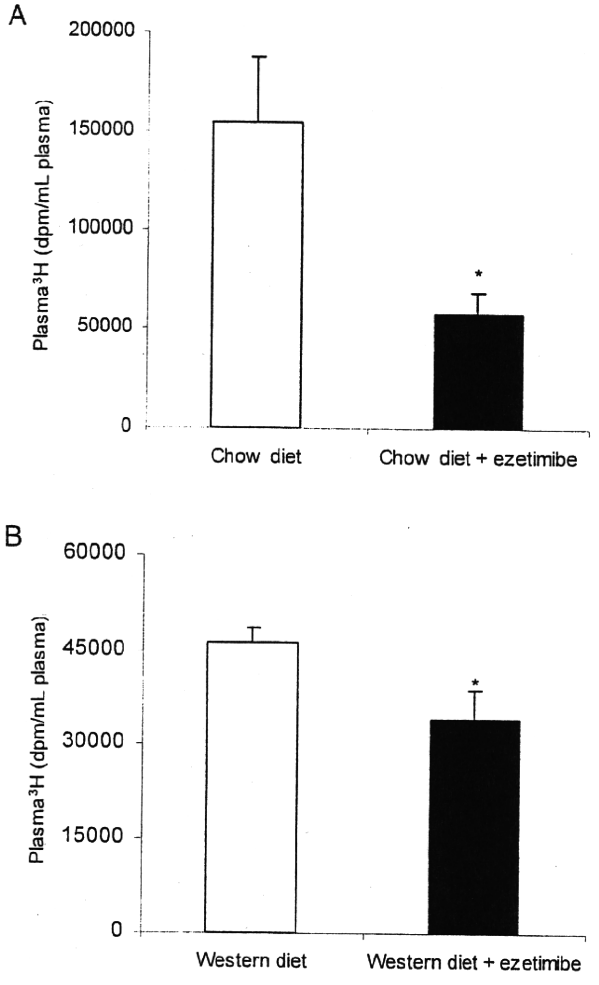

Fig. 4. Ezetimibe Reduces the Intestinal Absorption of Labeled Triolein.

Treated and non-treated mice from CD36KO (A) and WT mice (B) were loaded with 17 μL/g body weight of olive oil containing 3 μCi of [9,10-^{3}H(N)] triolein. Three hours after oral fat loading, ezetimibe reduced significantly ^{3}H radioactivity in plasma of both groups. ($^*p < 0.05$)

Effect of Ezetimibe on the Transcriptional Regulation of Genes Involved in Fatty Acid Transport, TG Formation and CM Assembly in the Intestinal Cells in the Postprandial State

To determine the molecular mechanisms involved in the attenuation of PHTG by ezetimibe, qRT-PCR using total mRNA isolated from the small intestines was performed, and the expression of genes associated with FA transport, TG formation and CM assembly in the intestine of both strains treated and non-treated with ezetimibe was examined.

In CD36KO mice, the mRNA levels of fatty acid transport protein 4 (FATP4), the only FATP in the intestine, were significantly reduced by the administration of ezetimibe, whereas the mRNA levels of fatty acid binding protein 1 (FABP1) and FABP-2, which are also associated with the transport of long-chain FAs, were not changed significantly in the treated groups. The mRNA expression of stearoyl-coenzyme A desaturase 1 (SCD1), diacyl glycerol acyl transferase 1 (DGAT1), DGAT-2, and monoacyl glycerol acyl transferase 2 (MGAT2), all involved in the intracellular formation of TG in intestinal epithelial cells, did not change significantly in the presence of ezetimibe. Interestingly, ApoB mRNA was found to be decreased in mice treated with ezetimibe; this reduction might be associated with a decrease in the expression of apobec-1 mRNA, one of the important factors and components of the protein complex involved in the mRNA edition of ApoB. The expression of microsome triglyceride transfer protein (MTP), which has an important role in CM assembly in intestinal cells, did not change significantly in the presence of ezetimibe. These results suggest that reduction in the hypertriglyceridemic response of ezetimibe in CD36KO mice might be associated with a decrease in cholesterol absorption, fatty acid transport and apo B48 synthesis, resulting in the attenuated formation of CM by a reduction of apoB48 mRNA (**Fig. 5A**).

In WT mice fed a western diet, the mRNA levels of FATP4 and FABP2 were found to be reduced by the administration of ezetimibe, while FABP1 and CD36 were unaffected by this treatment; we also found that SCD1, DGAT1 and DGAT2 were decreased in treated mice. Moreover, in this group, we also found that apoB mRNA was decreased, and this reduction might be associated with a decrease in ACF (apobec-1 complementary factor), a component of the apoB mRNA editing complex. These results suggest that ezetimibe reduces PHTG in WT mice by decreasing fatty acid transport, TG formation and CM assembly in intestinal epithelial cells (**Fig. 5B**).

We also identified an upregulation of fatty acid synthase (FAS), and acetyl-Coenzyme A acetyltransferase 2 (ACAT2) in both groups, which might be due to compensatory responses to the reduction of fatty acid transport and CM production.

Discussion

In the present study, we have investigated the inhibitory effect of ezetimibe on PHTG in MetS using two different animal models: WT mice fed a western, high fat, high cholesterol diet; and CD36KO mice, which is considered as a model of PHTG and a mono-

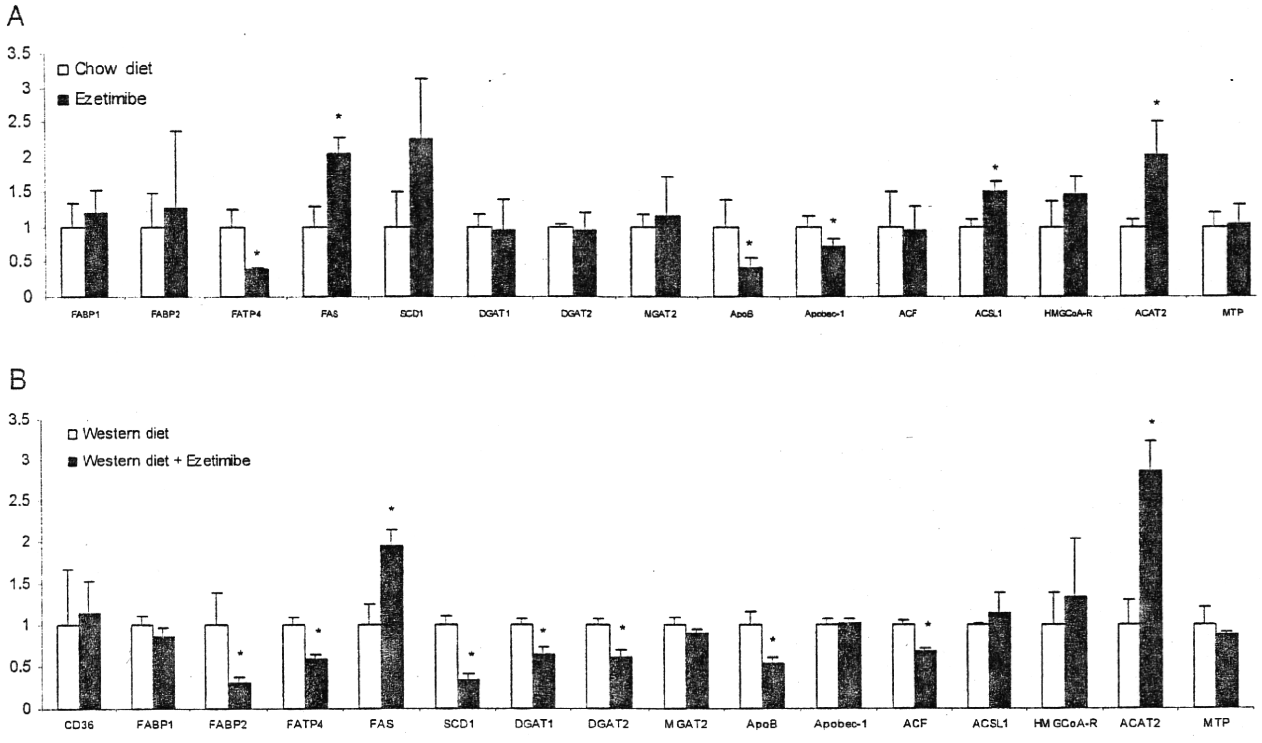

Fig. 5. Effect of Ezetimibe on the Transcriptional Regulation of Genes Involved in Fatty Acid Transport, TG Formation and CM Assembly in the Intestinal Cells in the Postprandial State.

We performed qRT-PCR using total mRNA isolated from intestines, and examined the expression of genes associated with FA transport, TG formation and CM assembly for CD36KO (A) and found that ezetimibe administration significantly inhibited the expression of FATP4, apoB and apobec1. In WT mice (B) ezetimibe decreased the expression of FATP4, FABP2, DGAT1, DGAT2, SCD1, apoB and ACF significantly. There was also the upregulation of FAS and ACAT2, which could correspond to a compensatory response. In both cases, ezetimibe decreased the expression of genes involved in FA metabolism and CM production.

genic model of MetS[11]. We have elucidated the possible molecular mechanisms responsible for the reduced production of ApoB-48-containing lipoproteins in intestinal epithelial cells. Because of the lack of hepatic NPC1L1 expression in mice[20], the usage of mice has an advantage to understanding the physiological mechanisms of lipid metabolism in the small intestines as a main target of ezetimibe, contrary to human subjects in which NPC1L1 is believed to be expressed in both small intestines and the liver. Ezetimibe is a strong inhibitor of cholesterol absorption via NPC1L1, and thus cholesterol incorporation into the CM synthesized in the small intestines is reduced by ezetimibe treatment. Therefore, the reduction of cholesterol content in CM and CM remnants may result in a decreased cholesterol pool in the liver, leading to the enhancement of hepatic LDL receptor. Thus, ezetimibe treatment may enhance the catabolism of LDL via hepatic LDL receptor, resulting in the reduction of LDL-cholesterol and possibly CM remnants. Furthermore, reduced cholesterol absorption may lead to the loss of the substrate for CM formation and thereby to attenuation of CM synthesis in the small intestines.

We found that, in both groups, WT mice fed a high fat diet and CD36KO mice fed a chow diet, ezetimibe did not reduce plasma cholesterol concentrations significantly in the fasting state (**Fig. 1B**); however, there remained a small, non-significant tendency for the cholesterol content in plasma to fall in both groups. This might be associated with increased endogenous production of cholesterol in both the intestine and liver in both models, possibly through an increased expression of HMG-CoA synthase, which should be further considered.

We also found that in WT mice fed a chow diet, ezetimibe did not decrease postprandial TG levels (**Fig. 1A**); however, when WT mice were fed a high-fat, high-cholesterol diet, ezetimibe reduced the PHTG to normal levels. This might suggest that ezetimibe could reduce postprandial triglyceride levels in

conditions of CM overproduction.

In a previous publication, our group found that CD36KO mice have an increased TG response to acute fat loads in both plasma and lymph[11]. In the current study, we found that CD36KO showed a higher TG concentration than WT mice even in a high fat loading state in intestinal lymph (**Fig. 3**); this might suggest the hypothesis that CD36KO mice would have a larger CM than WT mice. However, since the CM fraction in our HPLC method was included in the void volume, we could not determine the specific size of individual particles in this fraction, which is considered a whole group, and therefore, we were not able to confirm whether there was really a difference in particle size between these two groups.

The reduced absorption of long-chain FAs observed in this study was in part associated with an inhibitory effect on FATP4 in CD36KO mice as well as the reduction of both FATP4 and FABP2 intestinal expression in WT mice. FATP4 is the only FATP expressed in the intestines[21], is located in the ER of the intestinal cells and has demonstrated acyl-CoA synthetase activity, which decreases the intracellular concentration of FAs, and would indirectly increase FA uptake when the extracellular concentration is high enough, as in the postprandial state[22]. FATP4 has also been associated with obesity and the insulin-resistant state[23]. Labonté *et al.*[24] reported a reduction of the FATP4 amount in the intestines of both WT mice receiving ezetimibe and NPC1L1 knockout mice compared to WT controls. Although we did not measure the amount of FATP4 protein by Western blotting, we found a decrease in the mRNA content in both treated groups, suggesting inhibition of the regulation of FATP4 at the transcriptional level, which would lead to a decreased amount of FATP4. Taken together, these findings suggest a close relationship between the presence of active NPC1L1 and the uptake, intracellular transport and esterification of long-chain FAs.

In the current study, we also found that WT mice fed a western diet under ezetimibe treatment showed a reduced expression of DGAT1 and DGAT2, two proteins involved in TG synthesis, located in the ER[25], as well as a decreased expression of SCD1, which is an important lipogenic factor associated with dietary saturated fat-related obesity[26]. SCD1 has been reported to colocalize and interact with DGAT2[27], suggesting a mechanism of the incorporation of endogenously synthesized FAs into TG. Therefore, ezetimibe might also decrease PHTG in WT mice fed a western diet by reducing the formation of TG in intestinal cells.

Interestingly, in CD36KO mice, ezetimibe administration inhibited only FATP4 expression in the steps prior to CM assembly to reduce PHTG, but not FABP2, nor any of the proteins involved in TG production, as in WT mice, which might suggest that FATP4 could play an essential role in FA metabolism in the CD36KO model, different from WT mice, which also supports the idea that intestinal lipid metabolism in CD36KO mice is different from in WT mice.

On the other hand, we found that ezetimibe administration reduced ApoB mRNA in both treated groups, and moreover, ezetimibe decreased the mRNA levels of apobec1 in CD36KO mice and Apobec1 complementary factor (ACF) in WT mice. Whether ezetimibe decreased ApoB48 mass in lymph only by inhibiting the transcription or by enhancing the post-transcriptional degradation of ApoB is not known yet, and further examination will be required to gain a better understanding of intestinal ApoB metabolism.

Apobec1 is the catalytic subunit of the ApoB editing complex; in the absence of apobec1, there is no ApoB mRNA edition; apobec1 KO mice lack ApoB48, and the only ApoB found in this model is ApoB100[28]. In our study, ezetimibe decreased apobec1 mRNA significantly in CD36KO mice; however, we did not find any traces of ApoB100 in the intestinal lymph collected; therefore, we presume that ApoB mRNA edition was not so low as to make the enterocytes produce ApoB100-containing lipoproteins, but decreased enough to reduce the production of ApoB48 which, in addition to the presence of low TG as a substrate, led to reduced CM production.

Apobec1 complementary factor (ACF), the RNA-binding subunit of the editing complex, interacts with both apobec1 and ApoB mRNA, positioning the ApoB mRNA structure in the optimal configuration to expose the C residue to apobec1, and it has been proposed to be responsible for the specificity of the reaction[29], and a stabilizer for apobec1[30]. It has been proposed that ACF plays a pivotal role independent of apobec1, since attempts to generate ACF KO mice were not successful beyond the blastocyst state, and siRNA knockdown of ACF in rat and human cells induced an increase in apoptosis. In heterozygote ACF KO mice, ACF protein was found to be decreased in the small intestines; however, intestinal ApoB mRNA edition was not compromised[31]. From this evidence, we could not draw the conclusion that the lowering effect of ezetimibe on the expression of ACF would be actually relevant to ApoB mRNA edition and the production of CM in WT mice.

We have summarized in **Fig. 6** the possible

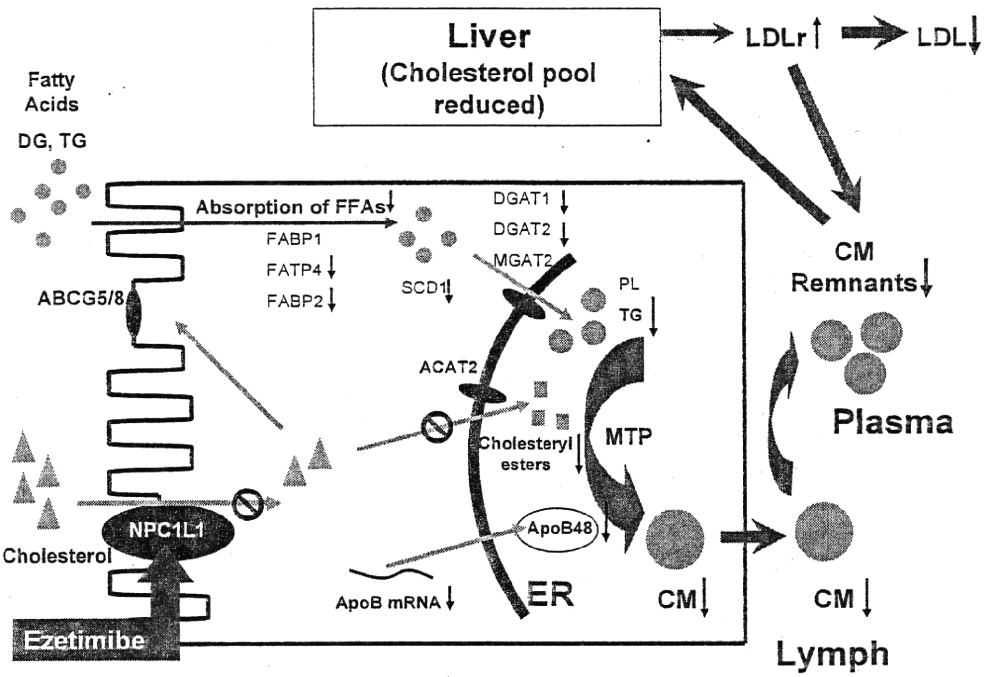

Fig. 6. Possible Mechanisms for the Inhibitory Effect of Ezetimibe on Postprandial Hypertriglyceridemia.

Administration of ezetimibe alone reduces PHTG by inhibiting cholesterol absorption and the expression of genes involved in the uptake, intracellular trafficking and metabolism of long-chain FAs (FATP4 in both WT and CD36KO mice and FABP2 in WT mice only), as well as by decreasing the formation of TG (SCD1, DGAT1 and DGAT2 in WT mice) and the expression of apoB (both WT and CD36KO mice), necessary for the production of ApoB48-containing lipoproteins in the small intestine. Furthermore, reduced cholesterol influx to the liver may lead to the up-regulation of hepatic LDL receptor, resulting in the enhanced catabolism of LDL and CM remnants.

mechanisms for the inhibitory effect of ezetimibe treatment on postprandial hypertriglyceridemia. The administration of ezetimibe alone reduces PHTG by inhibiting cholesterol absorption and the expression of genes involved in the uptake, intracellular trafficking and the metabolism of long-chain FAs (FATP4 in both WT and CD36KO mice and FABP2 in WT mice only), as well as by decreasing the formation of TG (SCD1, DGAT1 and DGAT2 in WT mice) and the expression of ApoB (both WT and CD36KO mice) necessary for the production of ApoB48-containing lipoproteins in the small intestine. Furthermore, reduced cholesterol influx to the liver may lead to the up-regulation of hepatic LDL receptor, resulting in the enhanced catabolism of LDL and CM remnants.

In conclusion, ezetimibe alone reduces PHTG in mouse models of MetS by inhibiting cholesterol absorption and uptake, intracellular trafficking and the metabolism of long-chain FAs, as well as decreasing the formation of TG and the expression of apoB, necessary for the production of apoB48-containing lipoproteins in the small intestine. Thus, ezetimibe strongly attenuates the intestinal production of CM, resulting in the inhibition of PHTG, which may eventually lead to the reduction of atherosclerosis in both animal models and humans.

Acknowledgments

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Original Article

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.

J Atheroscler Thromb, 2010; 17:610-618.

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

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

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density lipoproteins (VLDL) and their remnants, are atherogenic[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[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[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[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[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[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[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[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[16], and is activated by several synthetic compounds. Fenofibrate, a PPAR-α ligand, has been demonstrated to reduce TG levels in fasting and postprandial states in a cohort of hypertriglyceridemic subjects with MetS; this TG-lowering effect resulted primarily from reductions in fasting and postprandial concentrations of large and medium VLDL particles[17]. Moreover, fenofibrate has been shown to reduce non-fatal myocardial infarctions and coronary revascularizations in diabetic patients[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[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 μL/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)[20], where 200 μL of plasma or lymph were dissolved in loading buffer and loaded onto TSK gel Lipopropak XL columns. Triglyceride concentrations in the flow-through were measured continuously and simultaneously. The correspondence of 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℃.

Total RNA from tissue samples was extracted and purified using the RNeasy 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 transcripted 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'-atcaacaccaaccttaggcg-3' and 5'-aacccttgtctgggtgactg-3', FABP1 (NM_017399): 5'-catccagaaagggaaggaca-3' and 5'-ttttcccc-agtcatggtctc-3', FABP2 (NM_007980): 5'-ttgctgtccgag-aggtttct-3' and 5'-gctttgacaaggctggagac-3', DGAT-1 (NM_010046): 5'-gtgcacaagtggtgcatcag-3' and 5'-cag-tgggatctgagccatc-3', DGAT-2 (NM_026384): 5'-agtg-gcaatgctatcatcatcgt-3' and 5'-aaggaataagtgggaacca-gatca-3', MGAT-2 (NM_177448): 5'-gaagaagcagcat-cagggac-3' and 5'-gtgtgggattaggggactt-3', ApoB (NM_009693): 5'-tgggattccatctgccatctcgag-3' and 5'-gtaga-gatccatcacaggacaatg-3', MTTP (NM_008642): 5'-cat-gtcagccatcctgtttg-3' and 5'-ctcgcgataccacagactga-3', and GAPDH (NM_008084): 5'-actccactcacggcaaa-ttc-3' and 5'-tctccatggtggtgaagaca-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

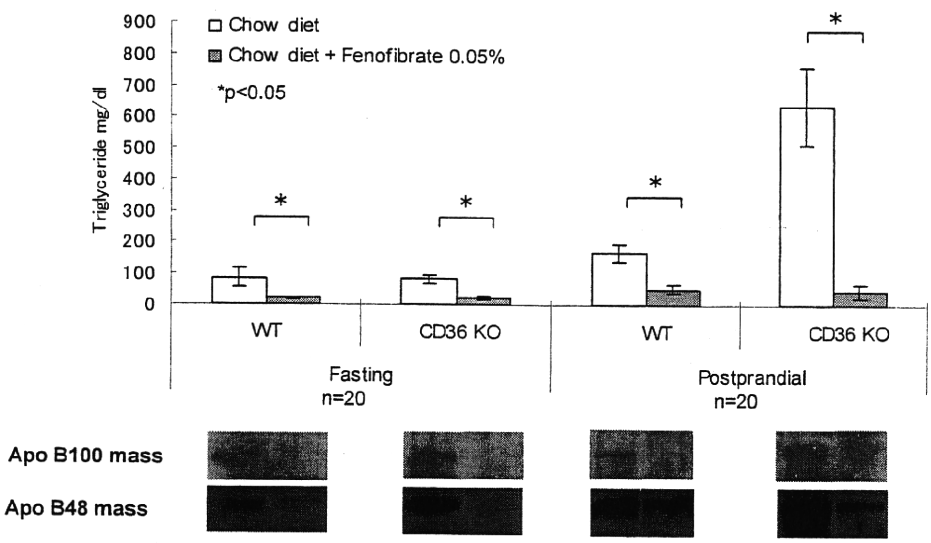

Fig. 1. Fenofibrate decreased plasma TG levels in CD36 knockout and WT mice in fasting and postprandial states.

(Upper panel) Addition of fenofibrate (gray) showed a significant decrease in TG levels in fasting and postprandial states in WT and CD36KO mice; the TG reductions for WT and CD36KO mice were 3.2 and 13.9 times, respectively. ($p < 0.05$)

(Lower panel) Representative Western blot images of apoB-100 and apoB-48 masses loaded the same amount of plasma in each subgroup.

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**).

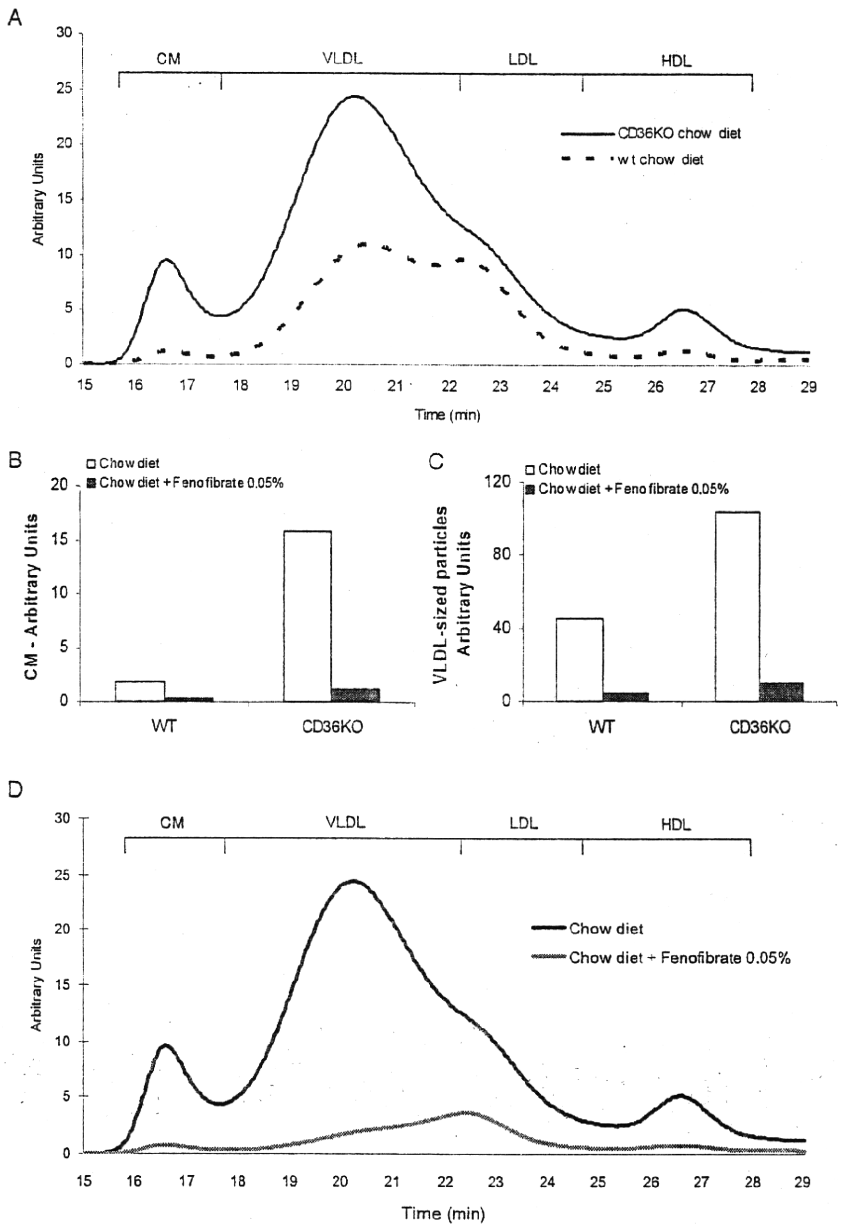

Fig. 2. Effects of fenofibrate on lipoprotein fractions in plasma of CD36KO mice in postprandial state.

(A) HPLC performed on plasma showed a higher 3-hour postprandial TG response of CD36KO (solid line) than WT mice (dashed line). Fenofibrate treatment (black) decreased postprandial plasma TG levels in CM (B) and VLDL-sized particles which also include CM remnants (C). (D) Plasma HPLC curves in postprandial state before (solid black line) and after (solid gray line) fenofibrate treatment in CD36KO mice

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

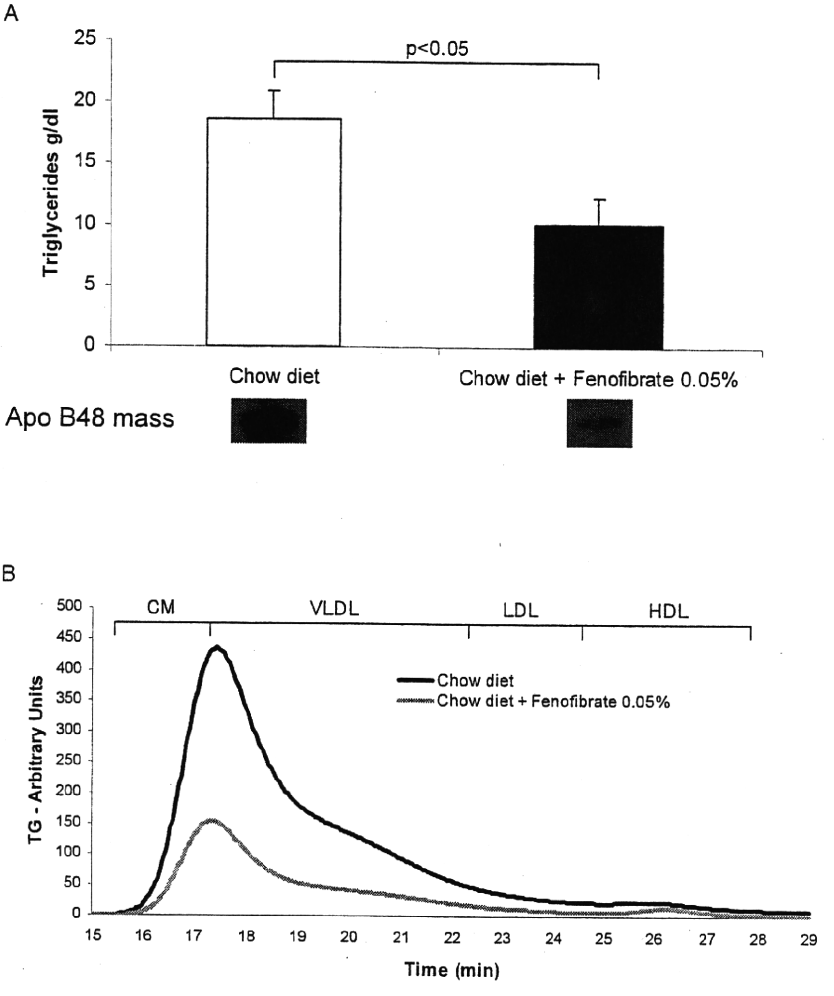

Fig. 3. Fenofibrate reduces postprandial TG and apoB-48 mass in intestinal lymph of CD36KO mice.

(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.

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 levels of fatty acid transport protein (FATP)-4, and fatty acid binding proteins (FABP)-1 and FABP-2, which are highly associated with the uptake and transport of long chain FAs, did not change significantly in the presence of fenofibrate. 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**).

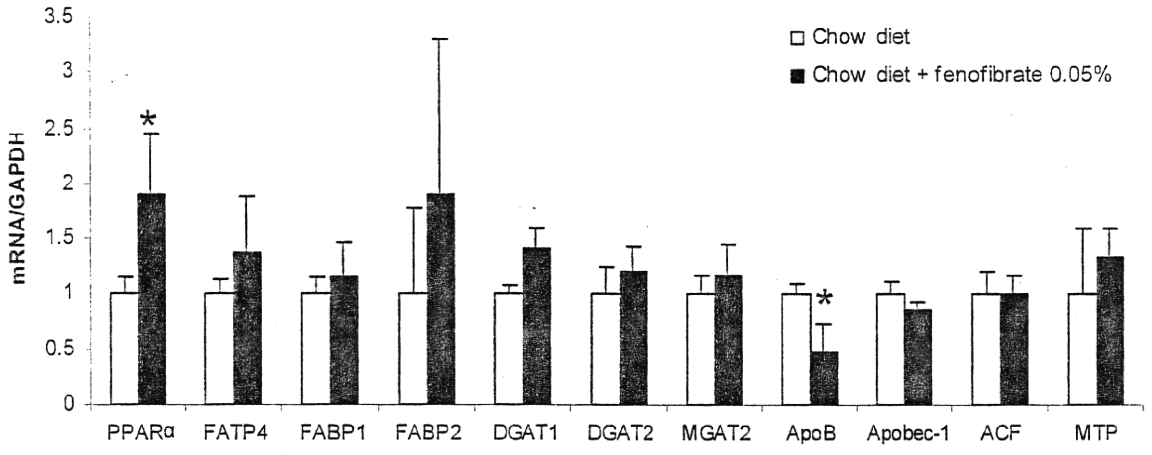

Fig. 4. Fenofibrate was involved in the transcriptional regulation of lipid metabolism-related genes in the intestine of CD36KO mice in postprandial state.

The mRNA expression of genes involved in intestinal TG manipulation and CM production were evaluated by qRT-PCR. Fenofibrate decreased apoB mRNA in CD36KO mice in postprandial state significantly ($p<0.05$). No significant difference was observed in the expression of genes that regulate apoB mRNA production, as well as in those associated with fatty acid transport, TG formation, and CM assembly.

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-