

Fenofibrate, a peroxisome proliferator-activated receptor α agonist, alters triglyceride metabolism in enterocytes of mice

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ABSTRACT

Fenofibrate, a drug in the fibrate class of amphiphathic carboxylic acids, has multiple blood lipid modifying actions, which are beneficial to the prevention of atherosclerosis. One of its benefits is in lowering fasting and postprandial blood triglyceride (TG) concentrations. The goal of this study was to determine whether the hypotriglyceridemic actions of fenofibrate in the postprandial state include alterations in TG and fatty acid metabolism in the small intestine. We found that the hypotriglyceridemic actions of fenofibrate in the postprandial state of high-fat (HF) fed mice include a decrease in supply of TG for secretion by the small intestine. A decreased supply of TG for secretion was due in part to the decreased dietary fat absorption and increased intestinal fatty acid oxidation in fenofibrate compared to vehicle treated HF fed mice. These results suggest that the effects of fenofibrate on the small intestine play a critical role in the hypotriglyceridemic effects of fenofibrate.

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1. Introduction

Fenofibrate, a drug in the fibrate class of amphiphathic carboxylic acids, has multiple blood lipid modifying actions, which are beneficial to the prevention of atherosclerosis [1,2]. Of these actions, lowering fasting and postprandial blood triglyceride (TG) concentrations are among its strongest effects [1,3]. In addition to elevated fasting blood TG concentration, elevated postprandial blood TG has been shown to be an accurate independent predictor of coronary artery disease [4]. The hypotriglyceridemic effects of fenofibrate are due to its ability to bind and activate the transcription factor, peroxisome proliferator-activated receptor α (PPAR α) [5]. Fenofibrate binding and activation of PPAR α results in a change in transcription in multiple genes involved in TG and fatty acid catabolism in many cell types; however, the major mechanism for its hypotriglyceridemic action is thought to be the improved clearance of TGs from circulation [2,6–9].

In the postprandial state, dietary fat contributes significantly to the postprandial triglyceridemic response. Dietary fat in the form of TGs

are hydrolyzed by pancreatic lipase in the intestinal lumen into monoglycerides and free fatty acids where they are incorporated into micelles for delivery to enterocytes for absorption. Within enterocytes, the monoglycerides and free fatty acids are re-esterified to form TGs, which under high dietary fat challenges are temporarily stored in cytoplasmic lipid droplets (CLDs) within enterocytes [10] as well as packaged in chylomicrons (CMs) for secretion via the lymphatic system into circulation [11]. The TG secreted into circulation in CMs is cleared from circulation by peripheral tissues due to postprandial upregulation of lipoprotein lipase (LPL), which hydrolyzes the TG to facilitate fatty acid uptake.

The hypotriglyceridemic effect of fenofibrate in the postprandial state may not only be due to the well established rapid clearance of TG from circulation due in part to increased LPL activity [6], but also due to the decreased secretion of TG into circulation from the small intestine. Evidence for a role for PPAR α and effects of fenofibrate on the small intestine are emerging. PPAR α is expressed in enterocytes along the length of the small intestine with highest levels in the duodenum and the jejunum, and higher levels in villus tips than in crypts [12,13]. This expression pattern is similar to that of several other genes involved in dietary fat absorption including microsomal triglyceride transfer protein (*Mttp*), diacylglycerol acyltransferase 1 (*Dgat1*), fatty acid translocase (*Cd36*), and fatty acid transport protein 4 (*Fatp4*) [14]. In microarray studies of intestinal mucosa, PPAR α activation results in altered expression of lipid metabolism and transport genes as well as genes in other intestinal functions such as

Abbreviations: CM, chylomicrons; CARS, coherent anti-Stokes Raman scattering; CLD, cytoplasmic lipid droplets; FEN, fenofibrate; HF, high-fat; LPL, lipoprotein lipase; PPAR α , peroxisome proliferator-activated receptor- α ; TG, triglyceride; VEH, vehicle

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immune and inflammatory response [12,13]. In addition, fenofibrate was recently shown to reduce cholesterol absorption via a PPAR α dependent decrease in NPC1L1 expression in intestinal mucosa in mice [15]. Finally, PPAR α activation also resulted in increased villus height and area in the jejunum in mice [12]. The effects of fenofibrate on TG and fatty acid metabolism in the small intestine and its potential for decreasing postprandial triglyceridemic response are currently unknown.

The goal of this study was to determine whether the hypotriglyceridemic actions of fenofibrate in the postprandial state may include alterations in TG and fatty acid metabolism in the small intestine. We hypothesized that the effects of fenofibrate on the small intestine may contribute to lower postprandial blood TG concentration by decreasing the supply of TG for secretion from the intestine into the bloodstream by decreasing dietary fat absorption and increasing intestinal fatty acid oxidation in high-fat (HF) fed mice.

2. Materials and methods

2.1. Mice and experimental design

All procedures were approved by the Purdue Animal Care and Use Committee. For all experiments, three to five month-old male mice (C57BL/6) were used. Mice were maintained in a specific pathogen-free barrier facility with a 12-h light/dark cycle (6 PM/6 AM) with free access to food and water. Mice were fed a HF diet (D12492, Research Diets, Inc., New Brunswick, NJ) for two weeks before treatment as well as during the treatment. The HF diet consisted of 20% of calories from carbohydrate (35% sucrose, 65% starch), 20% from protein, and 60% from fat (mostly lard). Prior to the HF diet, mice were on a low-fat, rodent chow (PicoLab 5053, Lab Diets, Richmond, IN), consisting of 62.1% of calories from carbohydrate (starch), 24.7% from protein, and 13.2% from fat. Mice were gavaged daily for five days between 12 PM and 1 PM, with 200 mg/ml fenofibrate (Sigma-Aldrich, St. Louis, MO) suspended in olive oil at a dose of 800 mg/kg of body weight. Mice were euthanized and tissues harvested after a two-hour fast; thus, mice were in a postprandial state at termination of the study. The small intestine was divided into six equal length segments and labeled S1–S6 (proximal to distal) in relation to the stomach.

2.2. Postprandial TG secretion

Blood for measuring baseline TG concentration was obtained after a four hour fast via submandibular bleed. Mice were then injected with or without 500 mg/kg Tyloxapol (T0307, Sigma-) into the intraperitoneal cavity to block serum lipase activity. After 30 min, mice were administered an oral gavage of 200 μ l olive oil and plasma collected up to four hours post-gavage. Plasma TG concentration was determined by Wako L-Type TG determination kit (Wako Chemical USA, Richmond, VA). Plasma lipoproteins were separated by agarose gel electrophoresis (Titan Gel Lipoprotein Kit, Helena Laboratories, Beaumont, TX) and stained with Fat Red 7B.

2.3. Dietary fat absorption

Mice were housed individually for the last three days during the five days of fenofibrate treatment, food intake was determined and feces were collected daily. The feces were dried for 2 h using an ANKOMRD dryer (ANKOM technology, Macedon, NY) and extracted via the Folch method. The analysis allows for the measurement of lipid extracted per gram of dried fecal samples. The extracted lipids were dissolved in chloroform/methanol and separated by thin layer chromatography using hexane:ether:acetic acid (80:20:1). Lipids were visualized using iodine vapor. Standards used were cholesterol

oleate (60 μ g), cholesterol (300 μ g), oleic acid (60 μ g), and olive oil for TG (3 μ l of 30 μ l/ml olive oil in chloroform).

2.4. Fatty acid oxidation

Fatty acid oxidation in the intestine (S2 and S3 representing the jejunum) was determined *ex vivo* via the ³H₂O release method. Micelles consisting of 10 μ Ci/ml ³H-oleate, 10 mM taurocholate, 1 mM carnitine and with or without 1 mM hydroxyphenylglycine in PBS were made. The intestine tissue was thoroughly cleaned with PBS and cut open longitudinally on a glass plate, incubated for two minutes in micelle solution and then thoroughly rinsed with PBS and mucosa scraped. The scraped mucosa was then incubated with 200 μ l of 1 mM carnitine in PBS for 15 min, then quenched with 10% TCA and neutralized with 6 M NaOH before passing through an anion exchange column (DOWEX 1x2-400, Sigma,). The columns were washed twice with water and the radioactivity of the eluate was determined by liquid scintillation counting. The radioactivity of the eluate was normalized to protein concentration in each reaction. Protein concentration was determined by BCA assay (Pierce, Rockford, IL). Fatty acid oxidation is reported as a fold change between fenofibrate and vehicle treated mice.

2.5. Gene expression

Total RNA was extracted from tissues (S1 representing the duodenum) with RNA STAT60 (Tel-Test, Friendswood, TX) and then DNase treated with Turbo DNA-free (Ambion, Austin, TX). cDNA was synthesized from 1 μ g Dnase treated RNA by AffinityScript QPCR cDNA using oligo dT and random hexamer primers (Stratagene, La Jolla, CA). SYBR green QPCR was performed using Mx3000P QPCR System (Stratagene) and Brilliant II SYBR green master mix (Stratagene). Post-PCR products were subjected to 1.5% agarose gel electrophoresis for imaging product size. The expression of each gene was normalized to 18 s rRNA and calculated with the comparative CT method. Primers used for this study are as shown in Table 1 and were all validated for efficiency and correct product size in cDNA from mouse intestinal mucosa.

Table 1
Primers used for quantitative real time PCR.

Gene	Primer sequences
18 s rRNA	F 5'-TTAGAGTGTTCAAA GCAGGCCCGA-3' R 5'-TCTTGGCAAATGCTTTCGCTCTGG-3'
<i>Aco</i>	F 5'-ATATTACGTACGCTTACCCCGG-3' R 5'-GGCAGGTCATTCAAGTACGACAC-3'
<i>ApoAIV</i>	F 5'-CCAGCTAAGCAATGCCAAGGA-3' R 5'-TGCTCCTGCAACTTCTGCATGTTTC-3'
<i>ATGL</i>	F 5'-TGGAAACCAAGGACCTGATGACCA-3' R 5'-AGATGCTACCCGCTCTGCTCTTCA-3'
<i>Cd36</i>	F 5'-ATTGTACTGGGAGTGGCGAGAA-3' R 5'-AACTGTCTGTAGACAGTGTGCCT-3'
<i>Cpt1a</i>	F 5'-TGTGGTGTCCAAGTATCTGGCAGT-3' R 5'-AACACCATAGCCGTCATCAGCAAC-3'
<i>Fabp1</i>	F 5'-AGTACCAATGACAGGCCAGGAGA-3' R 5'-GACAATGTCGCCCAATGTCATGGT-3'
<i>Fabp2</i>	F 5'-AGAGGAAGCTTGAGCTCATGACA-3' R 5'-TCGCTTGGCTCAACTCCTTCATA-3'
<i>Fatp4</i>	F 5'-AACCAGGGTCCCAACACAGAAAG-3' R 5'-AGTTCCTGGCACCTCAACACATA-3'
<i>Mttp</i>	F 5'-AGTGCAGTTCTCACAGTACCCGTT-3' R 5'-AGCATATCGTTCGTGGAAGGGA-3'
<i>Ppara</i>	F 5'-TCGCGTACGGCAATGGCTTTATCA-3' R 5'-AGCTTTGGGAAGGAGGAGGTTGCA-3'
<i>Ucp2</i>	F 5'-TGTCCTGCTTCCGATTGAAGGT-3' R 5'-TTTACCACATCTGAGGCTGGGCT-3'

2.6. Tissue imaging

For intact tissue imaging, *ex vivo* fresh tissues (5 mm) from small intestine (S2, representing upper jejunum) were placed in 3 mL Dulbecco's Modified Eagle's Medium (Gibco, Carlsbad, CA) supplemented with 20 mM HEPES, 100 U/ml penicillin–streptomycin (Gibco), and 10% fetal bovine serum. Tissues kept at 4 °C maintained good morphology over 5 h. Small intestine tissue was cut longitudinally and laid flat for luminal imaging. All tissues were imaged within 3 h after euthanasia. Coherent anti-Stokes Raman scattering (CARS) imaging were performed at a multimodal microscope. Pump and Stokes lasers were generated from 2 Ti:sapphire lasers (Tsunami, Spectra-Physics, Mountain View, CA), with a pulse width of 5 ps. These 2 lasers were time synchronized (Lock-to-Clock, Spectra-Physics), collinearly combined, and directed into a laser scanning confocal microscope (FV300/IX71, Olympus America, Center Valley, PA). A 60× water objective (N.A. = 1.2, Olympus) was used to focus the laser beams into the sample. The average powers of the pump and Stokes beams at the sample were 40 and 30 mW respectively. For imaging TGs, the pump laser and the Stokes laser were tuned to 14140 cm⁻¹ and 11300 cm⁻¹ respectively in order to generate a Raman shift of ~2840 cm⁻¹ that excites the symmetric CH₂ vibration. The forward-detected CARS (F-CARS) signals were collected using an air condenser (N.A. = 0.55). External photomultiplier tube (R3896, Hamamatsu, Japan) detector was used to receive the F-CARS signals.

2.7. Intestinal TG concentrations

Lipids in intestinal mucosa (S1, representing duodenum) were extracted by the hexane/isopropanol (3:2) method. Briefly, after homogenization of the mucosa with 1 M Tris–HCl (pH 7.4), hexane/isopropanol (3:2) and water were added to the sample and was incubated for 30 min with occasional mixing. The upper part containing lipids were transferred to a new tube. After evaporating the organic phase under nitrogen, lipids were dissolved in isopropanol. TG concentration was determined by Wako L-Type TG determination kit (Wako) and normalized to protein concentration (Pierce).

2.8. Data and statistics

All the data are shown as mean ± SEM. Statistical significance was determined with a two-tailed Mann–Whitney test ($P < 0.05$), unless stated otherwise.

3. Results

3.1. Fenofibrate decreases fasting TG concentration and increases liver weight in HF fed mice

C57BL/6, male mice were fed a HF, obesogenic diet for two weeks and then continued on the HF diet in combination with daily oral administration of fenofibrate for five days. To validate that fenofibrate was active in our model we monitored fasting plasma TG concentration and liver weight at the end of the five-day treatment. Consistent with known effects of fenofibrate, we found lower fasting plasma TG

concentrations in fenofibrate compared to vehicle treated mice ($P = 0.04$, $n = 7$ mice, Table 2). Also consistent with known effects of fenofibrate in the liver, we found higher liver weights ($P = 0.002$, $n = 11–12$ mice, Table 2) and increased mRNA levels of PPAR α and CPT1a (5.8 ± 0.9 and 4.2 ± 0.9 fold higher respectively, $P = 0.04$ and 0.02 , $n = 3–5$ mice) in fenofibrate compared to vehicle treated mice. During the five-day treatment period, we did not observe differences in body weights or epididymal fat pad weights (Table 2).

3.2. Fenofibrate decreases the postprandial triglyceridemic response in part by decreasing TG secretion from enterocytes in HF fed mice

To determine whether fenofibrate decreases the postprandial triglyceridemic response in HF fed mice, we measured plasma TG concentrations hourly from 0 to 4 h post oral administration of 200 μ l olive oil in vehicle and fenofibrate treated mice. We found a decreased postprandial triglyceridemic response as determined by area under the curve (210 ± 20 vs. 360 ± 30 mg h/dl, in fenofibrate vs. vehicle treated mice, respectively, $P = 0.03$, $n = 3$ mice, Fig. 1A). To determine whether fenofibrate alters postprandial lipid content of lipoprotein fractions, we separated lipoproteins in plasma from mice at 0, 1, 2, and 4 h post oral administration of 200 μ l olive oil via agarose gel electrophoresis followed by Fat Red 7B staining. We found higher lipid content in HDL and lower lipid content in CM and VLDL fractions of fenofibrate compared to vehicle treated mice (Fig. 1C). To determine whether fenofibrate altered postprandial TG secretion, we measured plasma TG concentrations before and at 2 and 4 h post oral administration of 200 μ l olive oil in vehicle and fenofibrate treated mice which had been administered a TG clearance inhibitor, Tyloxapol. We found that fenofibrate treatment decreased postprandial TG secretion as evidenced by a slower rate, and less accumulation of TGs in the plasma of fenofibrate compared to vehicle treated mice over 4 h post gavage (800 ± 100 vs. 1500 ± 200 mg/dl, in fenofibrate vs. vehicle treated mice respectively, $P = 0.02$, $n = 5–6$ mice, Fig. 1B).

3.3. Fenofibrate decreases dietary fat absorption in HF fed mice

To determine whether fenofibrate treatment alters dietary fat absorption, we determined the food intake, fecal excretion, and fecal fat content in mice during the last three days of the five-day fenofibrate treatment portion of the experiment. We found that the dried weights of feces excreted were similar, however the feces were visibly different and fecal lipid content was significantly higher in fenofibrate compared to vehicle treated mice ($8 \pm 1\%$ vs. $2.7 \pm 0.2\%$ in fenofibrate vs. vehicle treated mice respectively, $P = 0.01$, $n = 5$ mice, Table 2). To determine whether digestion of dietary fat was inhibited, we separated the fecal lipid species by thin layer chromatography and compared TG content in fenofibrate and vehicle treated mice (Fig. 2). The TG content in the feces of both fenofibrate and vehicle treated mice was almost undetectable; suggesting the presence of highly efficient TG digestion in both groups. The fecal lipid species which were more abundant in fenofibrate compared to vehicle treated mice included fatty acids, cholesterol and phospholipids. Higher levels of cholesterol and phospholipids excreted in feces in response to fenofibrate have been previously reported [15,16]. To determine

Table 2
Metabolic characteristics of fenofibrate and vehicle treated mice. C57BL/6, male mice were treated with either vehicle or fenofibrate for 5 days. Mice were fasted for 2 h for measuring fasting plasma TG ($n = 7$ mice), then euthanized for liver and epididymal fat pads weight measurements ($n = 11–12$ mice). Food intake and fecal weights were measured over the last 3 days of the treatment period ($n = 5$ mice). Fecal fat was determined on feces collected during the last 3 days of treatment ($n = 5$ mice). Data are represented as mean ± SEM. Data with asterisks are significantly different compared to the vehicle treated mice, $P < 0.05$.

Treatment	Fasting plasma TG mg/dl	Body weight g	Liver g	Epididymal fat pad g	Food intake g/day	Fecal weight g/day	Fecal fat percent
VEH	27 ± 2	31 ± 1	1.1 ± 0.1	2.1 ± 0.6	2.5 ± 0.1	0.23 ± 0.1	2.7 ± 0.2
FEN	21 ± 1*	29 ± 2	2.1 ± 0.3*	1.7 ± 0.4	1.7 ± 0.2	0.23 ± 0.1	8 ± 1*

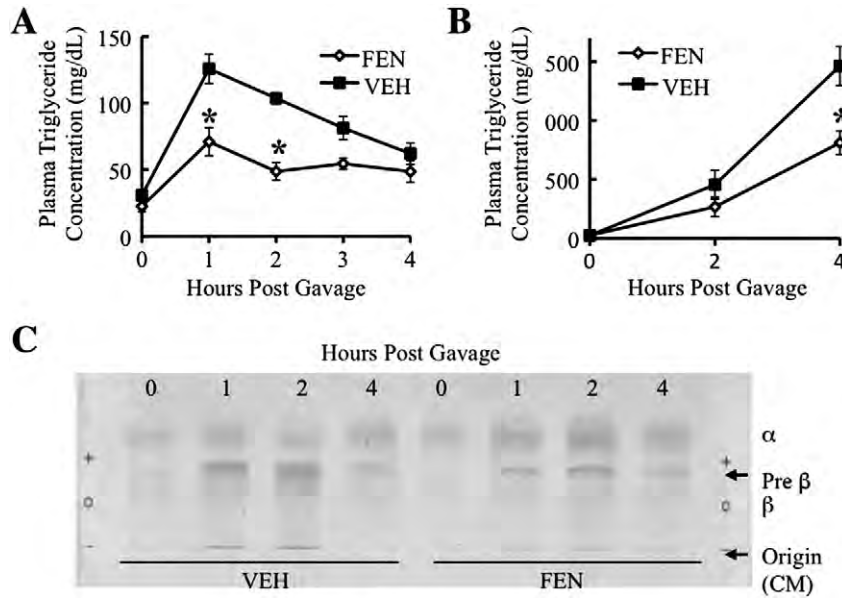


Fig. 1. Fenofibrate decreases the postprandial triglyceridemic response and TG secretion from enterocytes in HF fed mice. (A) Plasma TG concentration before and at 1, 2, 3 and 4 h after a 200 μ l olive oil challenge in fenofibrate and vehicle treated HF fed mice. Mice were fasted 4 h before olive oil administration. Data are represented as mean \pm SEM. Asterisks denote significant differences compared to vehicle treated mice, $P < 0.05$, $n = 3$ mice. (B) Plasma TG concentration before and at 2 and 4 h after 500 mg/kg Tyloxapol, a TG clearance inhibitor, and a 200 μ l olive oil challenge in fenofibrate and vehicle treated HF fed mice. Mice were fasted for 4 h before Tyloxapol and oral olive oil administration. Data are represented as mean \pm SEM. Asterisks denote significant differences compared to the vehicle treated mice, $P < 0.05$, $n = 4$ –5 mice. (C) Representative agarose gel of serum samples at 0, 1, 2 and 4 h post 200 μ l olive oil challenge in fenofibrate and vehicle treated HF fed mice.

whether the increased fatty acid excretion in feces was due to a defect in fatty acid absorption or trafficking by enterocytes, we quantified mRNA levels of *Mttp*, *Fatp4*, apolipoprotein AIV (*ApoAIV*) *Cd36*, Fatty acid binding protein1 (*Fabp1*), *Fabp2* and adipose triglyceride lipase (*ATGL*) in intestinal mucosa. We found trends of higher mRNA levels for *Mttp*, *Fatp4*, *ApoAIV* and *Cd36* in intestinal mucosa from fenofibrate compared to vehicle treated mice, which was contrary to the hypothesis that we would find lower levels of genes involved in fatty acids absorption (Fig. 3). Furthermore, we found a significant increase in *ATGL* and *Fabp2* mRNA expression and a decrease in *Fabp1* mRNA expression, between fenofibrate and vehicle treated mice ($P = 0.03$, 0.03 and 0.04, respectively, $n = 5$ –8 mice, Fig. 3).

3.4. Fenofibrate increases fatty acid oxidation in intestinal mucosa of HF fed mice

To determine whether fenofibrate increased expression of known PPAR α responsive genes and increased fatty acid oxidation in the small intestine, we measured mRNA abundance of PPAR α and PPAR α target genes in intestinal mucosa in HF fed fenofibrate and vehicle treated mice. Consistent with the well-known effects of fenofibrate on other peripheral tissues, fenofibrate treated mice had significantly higher mRNA levels of acyl-CoA oxidase (*Aco*) and uncoupling protein 2 (*Ucp2*) compared to vehicle treated mice ($P = 0.04$ and 0.02, respectively, $n = 6$ –8 mice, Fig. 4A). Although not significant we saw trends of higher *Ppar α* and carnitine palmitoyl transferase 1a (*Cpt1a*) mRNA levels in fenofibrate vs. vehicle treated mice ($P = 0.07$ and 0.13, respectively, $n = 5$ –8 mice, Fig. 4A). To determine whether these changes in gene expression correlated with fatty acid oxidation, we measured the production of $^3\text{H}_2\text{O}$ from ^3H -oleate in intestinal mucosa sections representing the jejunum (S2 and S3, defined in methods section). We found that fatty acid oxidation increased 1.5 fold in

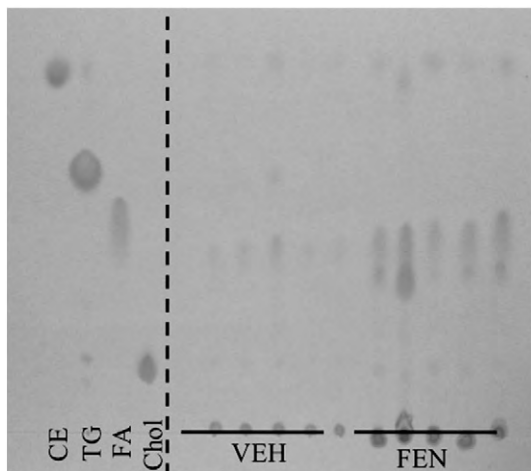


Fig. 2. Fenofibrate increases fecal fatty acids, cholesterol and phospholipids. Fecal lipid composition was determined by separating lipid species by thin layer chromatography using hexane:ether:acetic acid (80:20:1) and lipids detected via iodine, $n = 5$ mice. Standards used were cholesterol ester (CE), cholesterol (Chol), oleic acid for fatty acid (FA), olive oil for triglyceride (TG).

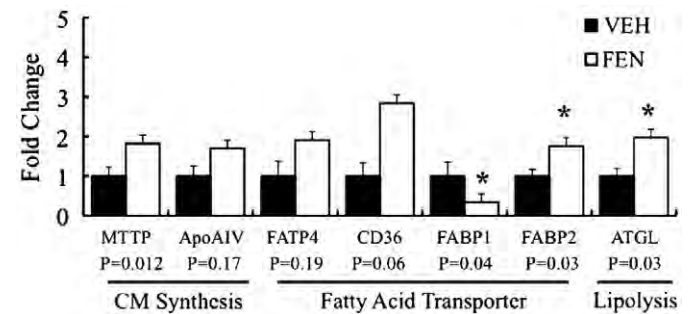


Fig. 3. Fenofibrate does not decrease mRNA levels of genes known to promote dietary fat absorption. QPCR analysis of genes involved in TG metabolism in the duodenum (S1) of fenofibrate and vehicle treated mice. Data are represented as mean \pm SEM. Bars with asterisks are significantly different compared to the vehicle treated mice, $P < 0.05$, $n = 6$ –8 mice.

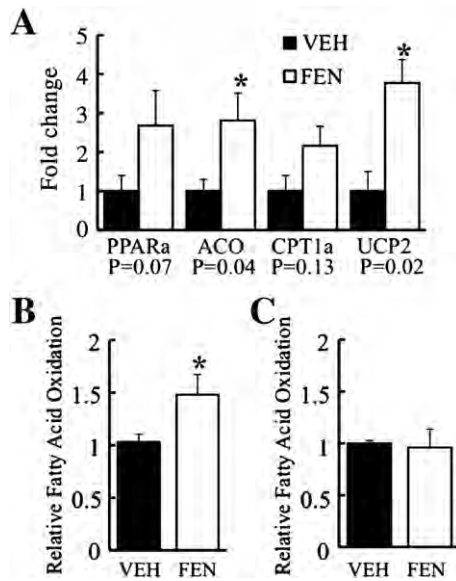


Fig. 4. Fenofibrate increases fatty acid oxidation in intestinal mucosa of HF fed mice. (A) QPCR analysis of genes involved in fatty acid oxidation in the duodenum (S1) of fenofibrate and vehicle treated HF fed mice. Data are represented as mean \pm SEM. Bars with asterisks are significantly different compared to the vehicle treated mice, $P < 0.05$, $n = 6-8$ mice. (B) Relative fatty acid oxidation in the jejunum (S2 and S3) *ex vivo* of fenofibrate treated mice normalized to vehicle treated HF fed mice. Data are represented as mean \pm SEM. Bars with asterisks are significantly different compared to the vehicle treated mice, $P < 0.05$, $n = 11-12$ mice. (C) Relative fatty acid oxidation in lower jejunum (S4) *ex vivo* of fenofibrate treated mice normalized to vehicle treated mice with hydroxyphenylglycine, an inhibitor of fatty acid oxidation, $n = 4$ mice.

fenofibrate compared to vehicle treated HF fed mice ($P = 0.02$, $n = 11-12$ mice, Fig. 4B) in this region of the small intestine. To demonstrate that we were indeed measuring fatty acid oxidation, we measured fatty acid oxidation in an adjacent section of the small intestine in the presence of hydroxyphenylglycine, an inhibitor of mitochondrial fatty acid oxidation as a control. We found no differences in fatty acid oxidation in fenofibrate compared to vehicle treated HF fed mice under these conditions (Fig. 4C). The results

suggest that the increase in fatty acid oxidation may be due to increases in mitochondrial fatty acid oxidation; however, a more thorough analysis is needed to determine the contribution of peroxisomal vs. mitochondrial fatty acid oxidation in the intestine in response to fenofibrate.

3.5. Fenofibrate decreases TG storage in enterocytes of HF fed mice

To determine whether fenofibrate affected TG storage in the small intestine we used coherent anti-Stokes Raman scattering (CARS) microscopy to image the upper jejunum (S2) and biochemically determined the amount of TG in adjacent intestinal mucosa sections. CARS microscopy is an emerging technique that permits vibration imaging of specific molecules with three-dimensional submicron spatial resolution. A strong CARS signal is generated from C–H stretch vibrations allowing label- and fixation-free imaging of lipids rich in fatty acids. We found abundant TG stored CLD in enterocytes of vehicle treated mice as expected (Fig. 5A and B, representative images from 3 mice) and little TG stored in CLD of fenofibrate treated mice. Consistent with CARS imaging results, biochemical analysis of TG in intestinal mucosa showed lower TG concentration in fenofibrate compared to the vehicle treated mice (0.05 ± 0.01 vs. 0.09 ± 0.03 mg of TG/mg of protein in fenofibrate vs. vehicle treated mice respectively, $P = 0.03$ $n = 6-9$ mice, Fig. 5E). The response to fenofibrate on TG storage in the intestine is similar when determined via CARS microscopy and biochemical analysis; however, the magnitude of the response differs. CARS signals are quadratic with respect to concentration, but the advantage of CARS images is that it allow for direct observation of TG storage in cytoplasmic lipid droplets with in enterocytes. The biochemical analysis is linear with respect to concentration, but the intestinal mucosa isolated for biochemical analysis has the potential for contamination by mesenteric fat and TG present in the secretory pathway and lymphatics.

4. Discussion

We found that the hypotriglyceridemic actions of fenofibrate in the postprandial state of HF fed mice include alterations in TG and fatty acid metabolism in the small intestine. We found decreased

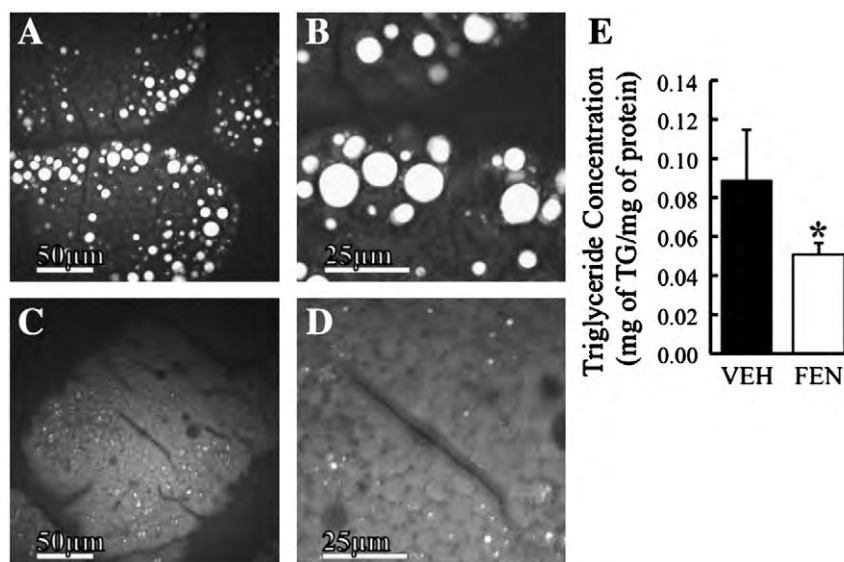


Fig. 5. Fenofibrate decreases TG storage in enterocytes of HF fed mice. C57BL/6, male mice were fed a HF diet for two weeks and then continued on the HF diet in combination with daily oral administration of fenofibrate for five days. Mice were fasted for two hours before euthanasia. Representative images ($n = 3$ mice) of TG storage in CLDs in of enterocytes in the upper jejunum (S2) of vehicle (A and B) and fenofibrate (C and D) treated mice using $60\times$ objective at $1\times$ and $3\times$ magnification. (E) TG concentration in intestinal mucosa representing the duodenum (S1) of the same mice described previously determined by biochemical extraction and colorimetric analysis. Data are represented as mean \pm SEM. Bars with asterisks are significantly different compared to the vehicle treated mice, $P < 0.05$, $n = 6-9$ mice.

dietary fat absorption and increased intestinal fatty acid oxidation, which decreased the supply of TG for secretion into circulation from the small intestine in fenofibrate compared to vehicle treatment in mice. Furthermore, we found less TG stored in CLDs in enterocytes of fenofibrate compared to vehicle treated HF fed mice. These findings demonstrate that the effects of fenofibrate on intestinal TG and fatty acid metabolism contribute to lower postprandial triglyceridemic response in HF fed, fenofibrate treated mice. In addition, these results reveal critical alterations in TG and fatty acid metabolism by fenofibrate in the small intestine, which allow for more favorable postprandial blood lipid profiles and may aid in the prevention of atherosclerosis.

Our results are consistent with previous studies demonstrating a reduced postprandial triglyceridemic response with fenofibrate treatment [1,3]. In response to a meal containing dietary fat, blood TG concentration initially increases, peaks, and then decreases over time [17,18]. Blood TG concentration at any time point is reflective of the balance between TG secretion into and clearance from circulation. At early time points after a HF meal, the majority of TG secreted into circulation is from dietary fat absorption via enterocytes. From the enterocytes, TG is secreted on CMs into circulation via lymphatics and ultimately cleared from circulation by the actions of LPL and fatty acid uptake by peripheral tissues. The contribution of TG by the liver during the postprandial state is minimal due to direct insulin action which dampens hepatic VLDL secretion [19].

The results presented here strongly indicate that the hypotriglyceridemic effect of fenofibrate in the postprandial state is not only due to the well established rapid clearance of TG from circulation [2,5], but also due to the decreased TG secretion into circulation during the postprandial state. We determined TG secretion after the administration of lipase inhibitor, Tyloxapol and of olive oil via oral gavage. Under these conditions, blood TG concentrations are primarily reflective of TG secretion from the intestine due to the large influx of dietary fat and the inhibition of VLDL secretion from the liver via insulin [19]. We further confirmed that secretion of intestinal TG on CMs is decreased by separating lipoproteins in plasma via agarose gel electrophoresis and staining for lipid present in lipoprotein fractions. These results are consistent with results from Sandoval et al. which recently showed that fenofibrate treatment of mice reduces postprandial CMs and VLDL in plasma [3]. In support of the decrease in TG secretion due to fenofibrate actions on the intestine, Bijland et al. recently reported that mice treated with fenofibrate have increased hepatic TG secretion in the fasted state than untreated mice [20]. These results contradict previous findings of the effects of fenofibrate on VLDL secretion from HepG2 cells showing decreased secretion [8]. These results; however, are in line with the important role of PPAR α in managing energy stores in the fasted state [8,21].

We investigated whether the decrease in postprandial TG secretion was due to a decrease in quantitative dietary fat absorption. We determined quantitative dietary fat absorption using a gravimetric approach and found statistically significant, but not physiologically dramatic, higher levels of fecal lipids in fenofibrate compared to vehicle treated HF fed mice (Table 2). Previous studies demonstrated increased fecal neutral sterol excretion in fenofibrate compared to vehicle treated mice [15]; however, the effects of fenofibrate on other fecal lipids are unknown. We followed this up with a semi-quantitative analysis of fecal lipids via thin layer chromatography. If digestion of TGs was inhibited, we would expect to see an increase in excretion of TG; but this was not the case as TG was barely detectable in lipid extracts of feces from either fenofibrate or vehicle treated mice (Fig. 2). However, we observed higher levels of free fatty acids, phospholipids and cholesterol in the feces of fenofibrate compared to vehicle treated mice. The higher levels of fecal free fatty acids were not due to decreased mRNA levels of genes involved in fatty acid uptake and metabolism in enterocytes. In fact, for most of the genes analyzed we found a trend towards higher and not lower mRNA levels

in intestinal mucosa of fenofibrate compared to vehicle treated mice. FABP1 was the only gene we found to have lower levels of mRNA in intestinal mucosa of fenofibrate compared to vehicle treated mice. Interestingly, FABP1-deficient mice do not exhibit fat malabsorption [22]. The higher levels of fecal cholesterol were also not surprising as previous reports demonstrated a defect in cholesterol absorption due to decreased NPC1L1 by fenofibrate [15]. Although the analysis of fecal lipid species in these experiments is only semi-quantitative, it is important to highlight that fecal excretion of fatty acids in mice is generally in milligram amounts whereas cholesterol excretion is in microgram amounts per day. Possible alterations in emulsification and micelle formation in fenofibrate treated mice may be present as well. Previous studies have demonstrated that fenofibrate alters bile content and/or secretion in a PPAR α dependent manner [15,16]. Further studies are necessary to understand whether these changes in bile are involved in the reduced absorption of dietary fat observed in our study.

The established effects of fenofibrate and PPAR α in regulation of fatty acid oxidation in liver and other tissues led us to question the role of fenofibrate in intestinal fatty acid oxidation. The small intestine expresses PPAR α and the machinery for fatty acid oxidation [12,13]. Other groups found that acute feeding of putative ligands for PPAR α such, eicosapentaenoic acid, docosahexaenoic acid and oleic acid, increased mRNA levels of genes related to fatty acid oxidation and fatty acid oxidation activity in the small intestine [13,23,24]. In this study, mRNA levels of genes involved in fatty acid oxidation were increased in the small intestine in response to fenofibrate (Fig. 4). Interestingly, the increase in FABP2 mRNA levels in the intestinal mucosa may be an indication of increased trafficking of fatty acids for oxidation in enterocytes of fenofibrate treated mice as was found in human intestinal epithelial cells with FABP2 overexpression [25]. We also found an increase in ATGL mRNA levels in the intestinal mucosa of fenofibrate vs. vehicle treated mice. ATGL is a lipase which hydrolyzes TGs in cytoplasmic lipid droplets. In hepatocytes, overexpression of ATGL results in higher levels of fatty acid oxidation [26]. Moreover, *ex vivo* studies resulted in higher fatty acid oxidation in the small intestine of fenofibrate compared to vehicle treated mice (Fig. 4). These results are consistent with the results found previously in other tissues [27–29].

Increased fatty acid catabolism within enterocytes has the potential to decrease the amount of TG secreted into circulation. However, the quantitative contribution of the increase in intestinal fatty acid oxidation found here towards the hypotriglyceridemic effect of fenofibrate is still unclear. In this study, we specifically determined fatty acid oxidation of fatty acids entering from the apical side of enterocytes. Previously, a difference in the fate of fatty acids for oxidation was dependent upon whether the fatty acids entered from the apical or basolateral side of the enterocyte with greater oxidation from basolateral entry [30]. This is interesting because PPAR α activation is a fasting response, which is known to increase lipolysis in the adipose tissue and export free fatty acids into circulation; therefore, we reason that fatty acids from the adipose tissue may also be substrate for intestinal fatty acid oxidation.

PPAR α activation is characterized by a reduction in tissue TG content, as seen in muscle and adipose tissue of mice [27,31]. Reduction in TG storage in the muscle has been attributed to an increase in fatty acid oxidation, which results in improved insulin sensitivity [27]. Previously, we reported dynamic storage of TG in CLDs in enterocytes in response to both acute and chronic HF feeding [10]. It is unclear whether TG accumulation in enterocytes affects their function including nutrient absorption and barrier roles. Fenofibrate, consistent with its action on muscle, reduced TG storage in enterocytes of the small intestine of HF fed mice (Fig. 4). From results presented here, the reduction of TG storage in enterocytes from fenofibrate treated mice, despite decreased TG secretion, can mechanistically be explained in part by the decrease in dietary fat

absorption and the increase in fatty acid oxidation. These results suggest that the effects of fenofibrate on the small intestine play a major role in the hypotriglyceridemic effects and have the potential to improve intestinal function.

Previous studies have also found that fenofibrate may be beneficial for the treatment of obesity and obesity associated insulin resistance, through increased TG clearance from circulation and increased fatty acid oxidation in the liver and muscle [6,27,32]. Although this study was not designed to investigate the effects of fenofibrate on weight management or obesity, or related insulin resistance, we speculate that the effects of fenofibrate on the small intestine may also play a positive role in weight management, obesity, and related insulin resistance by decreasing the energy intake portion of the energy balance equation via decreasing systemic availability of dietary fat, the most energy dense nutrient. Whether fenofibrate reduces food intake or has no effect on food intake is controversial [6,32,33]. A decrease in food intake in response to fenofibrate may be due to several mechanisms. We speculate that altering TG and fatty acid metabolism in enterocytes may also alter gut signaling and affect satiety. In fact, decreasing fatty acid oxidation in rats through administration of mercaptoacetate results in increased food intake. Although this result was initially thought to be due to actions on the liver, recent reports demonstrate that the increased food intake in response to decreased fatty acid oxidation is due to signals from the small intestine via abdominal vagal fibers [34]. Understanding the contributions of TG metabolism in enterocytes of the small intestine in dietary fat absorption, and potentially its direct effects on energy balance and food intake, will provide a better understanding to the pathology of metabolic disorders.

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