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Fenofibrate, a peroxisome proliferator-activated receptor α ligand, prevents abnormal liver function induced by a fasting–refeeding process

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ABSTRACT

Fenofibrate, a peroxisome proliferator-activated receptor α (PPARα) agonist, is an anti-hyperlipidemic agent that has been widely used in the treatment of dyslipidemia. In this study, we examined the effect of fenofibrate on liver damage caused by refeeding a high-fat diet (HFD) in mice after 24 h fasting. Here, we showed that refeeding HFD after fasting causes liver damage in mice determined by liver morphology and liver cell death. A detailed analysis revealed that hepatic lipid droplet formation is enhanced and triglyceride levels in liver are increased by refeeding HFD after starvation for 24 h. Also, NF-κB is activated and consequently induces the expression of TNF-α, IL1-β, COX-2, and NOS2. However, treating with fenofibrate attenuates the liver damage and triglyceride accumulation caused by the fasting–refeeding HFD process. Fenofibrate reduces the expression of NF-κB target genes but induces genes for peroxisomal fatty acid oxidation, peroxisome biogenesis and mitochondrial fatty acid oxidation. These results strongly suggest that the treatment of fenofibrate ameliorates the liver damage induced by fasting–refeeding HFD, possibly through the activation of fatty acid oxidation.

1. Introduction

Metabolic disorders, such as obesity, insulin resistance, and non-alcoholic fatty live disease caused by consuming a high-calorie diet, affect healthy life in developed countries [1]. Overnutrition potentially disturbs the nutrient homeostasis in human body and elicits chronic metabolic disorders [2]. Irregular eating habit, such as skipping meals or prolonged fasting, can also be a risk factor for metabolic syndrome [3–5]. Particularly, liver which is the major organ for controlling nutrient and energy homeostasis is affected by food availability [6,7]. During prolonged fasting, free fatty acids released from adipose tissue are mobilized into liver and induce an abrupt change in the expression of genes involved in hepatic lipid and glucose metabolism [8]. Refeeding a high-carbohydrate and fat-free diet after fasting stimulates lipogenic gene expression and reactive oxygen species (ROS) production in hepatocytes [9]. Inconsistent food availabilities, such as overnutrition or meal irregularity, are associated with metabolic syndrome but the underlying molecular mechanism is largely unknown.

Peroxisome proliferator-activated receptor α (PPARα) is a ligand-activated nuclear hormone receptor that controls lipid metabolism [10,11]. PPARα is highly expressed in various tissues with high fatty acid oxidation rate, particularly in liver which regulates fatty acid catabolism [12]. In physiological condition, fasting stimulates PPARα activity to enhance the expression of genes involved in fatty acid uptake, mitochondrial fatty acid oxidation, peroxisomal fatty acid oxidation, peroxisome proliferation, and ketogenesis [13–15]. There are several endogenous ligands for PPARα activation such as fatty acids and fatty acid metabolites, whereas fibrates, including fenofibrate and clofibrate, are well known pharmacological PPARα agonists that are widely used in the treatment of dyslipidemia [16,17]. In addition to its effects on lipid metabolism, PPARα also affects inflammatory signaling pathway by directly interacting with nuclear factor kB (NF-kB) to block inflammatory gene expression [18].

In the present study, we aimed to analyze the effects of fenofibrate treatment on a fasting–refeeding model which mimics irregular eating habit in hepatic lipid metabolism. Therefore, mice were fasted for 24 h, and then refed high fat diet for 16 h with or without twice injections of fenofibrate. Analysis on the liver tissues from mice subject to the fasting–refeeding process showed ballooning injury, neutral lipid accumulation, and NF-κB activation, implicating increased lipid mobilization to liver from nutrient. In contrast, fenofibrate treatment ameliorated the

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refeeding-mediated liver damage and reduced intrahepatic fat storage by inducing expression of genes closely related to fatty acid oxidation. Taken together, these findings indicate that a fasting–refeeding process impairs liver function potentially by accumulating triglycerides (TG) in hepatocytes, whereas activation of PPARα by fenofibrate treatment prevents the intrahepatic lipid storage by increasing fatty acid oxidation.

2. Materials and methods

2.1. Animal treatment

Eight week-old mice C57BL/6 mice were purchased from Orient Bio (Seongnam, Korea). Maintenance of mice and the experimental protocol were followed according to a standard animal protocol approved by the Animal Care and Use Committee at Wonkwang University. Mice were maintained for a week on a standard commercial diet (Research Diet Inc. USA) and then divided into four groups: fed ($n = 6$), fed+feno ($n = 6$), refed ($n = 6$) and refed+feno ($n = 6$). Both fed+feno and refed+feno groups of mice were received two times of fenofibrate (50 mg/kg Sigma), 5 h prior to fasting and 5 h prior to refeeding, by intraperitoneal injection. Fed and refed groups were injected with DMSO as a control. Fed mice were given a normal chow diet (carbohydrate 65%, protein 20%, fat 5%) whereas refed mice were deprived of food for 24 h but were allowed free access to water. After 24 h, the animal were given high fat diet (HFD, carbohydrate 20%, protein 20%, fat 60%) with access of water for 16 h. The animals were sacrificed and blood was collected and allowed to clot for 20 min at room temperature. Serum was then separated by centrifugation at 2000 g for 20 min and was stored at −80 °C until analysis. Liver was removed from each mouse, weighed and frozen in liquid nitrogen and stored at −80 °C until analysis or fixed with formalin. Tissues sections (5 μm thick) were used for hematoxylin and eosin (H&E), immunohistochemistry (IHC).

2.2. Measurement of hepatic triglyceride (TG) levels

Serum TG levels were measured by a 7600 Clinical Analyzer (Hitachi). For hepatic TG level, 50 mg of liver tissue was homogenized in 1 ml of chloroform–methanol mixture (2:1), centrifuged at 14,000 rpm for 15 min. The supernatant was dried and 200 μl of PBS was added to the sample. TG levels were measured according to the manufacturer's standard protocol (BioVision Incorporated, CA).

2.3. Histological and Immunohistochemical analysis

For histological analysis, the liver tissues fixed in 10% formalin solution and embedded in paraffin were sectioned. 5 μm thick sections were de-paraffinized in xylene and rehydrated through graded concentrations of ethanol. Hematoxylin and eosin (H&E) staining was performed using a standard protocol. For immunohistochemistry for NF-κB, TNF-α, and COX2 (Santa Cruz Biotechnology, CA), the sections were incubated with anti-NF-κB, anti-TNF-α or anti-COX2 antibodies and processed with biotin linkage and a streptavidin kit according to the manufacturer's instructions (LSAB System-HRP; Dako, Carpinteria, CA). Slides were visualized using a DAB chromogen substrate solution and counterstained with hematoxylin solution. Images were collected and analysed using a light microscope (IX71, Olympus, Tokyo, Japan). Slides were scored in a blinded manner.

2.4. BODIPY staining

Cryosectioned liver samples were fixed with paraformaldehyde for 20 min and washed with PBS. Glycine (2 mg/ml) was added to the samples for quenching auto-fluorescence from the section for 10 min. Then, sections were washed with PBS and stained with BODIPY 493/503 (Invirogen) at a final concentration of 30 μg/ml for 20 min in the dark. Sections were washed twice with PBS and stained with DAPI at 5 μg/ml for 10 min in the dark. After mounting the coverslip, the cells were observed using Olympus Flouview 1000 confocal laser scanning system.

2.5. TUNEL assay

Apoptosis in the liver was detected using a TUNEL assay kit according to the manufacturer's instructions (Roche Diagnostics, Germany). The frequency of apoptotic cells in sections was quantified by determining the percentage of TUNEL-positive cells in 3 random microscopic fields per specimen by fluorescence microscopy (IX71, Olympus, Tokyo, Japan).

2.6. Western blot analysis

To determine the expression levels of target proteins, western blotting was performed. Briefly, liver tissues were homogenized with lysis buffer (10 μmol/l Tris–HCl pH 7.6, 150 μmol/l NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 μmol/l EDTA, 50 μmol/l b-glycerophosphate, 1 μmol/l dithiothreitol, 1 μmol/l NaF, 1 μmol/l Na_3VO_4, 1 μmol/l phenylmethanesulfonylfluoride, and 1X protease inhibitor cocktail) and centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was taken. After measuring protein concentration (Bradford assay, Bio-rad), 30 μg of lysates was subject to electrophoresis on 10% SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and then transferred onto a nitrocellulose membrane. The proteins were visualized by a chemiluminescent solution according to the manufacturer's instructions (Thermo Scientific, Rockford, IL). Antibodies to ACOX1 (ABGENT, SAN DIEGO), DBP (OriGene Technologies, Rockville), and Catalase (Rockland Immunochemicals Inc. Gilbertsville) were used. The sample size for each group was three.

2.7. Quantitative RT-PCR analysis

Total RNA was isolated from liver tissue with the use of the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was subject to real-time PCR analysis with the use of SYBR Green (Roche Diagnostics, Germany). The sequences of primers for mouse cDNAs (forward and reverse, respectively) were as follows:

Acadvl, 5′- GGTGGTTTGGGCCTCTCTA-3′ and 5′-GGGTAACGCTAACACCAAGG-3′; Acadl, 5′-GCTTATGAATGTGTGCAACTCC-3′ and 5′-CCGAGCATCCACGTAAGC-3′; 36B4, 5′-CACTGGTCTA GGACCCGAGAAG -3′ and 5′-GGTGCCTCTGGAGATTTTCG-3′; ACOX1, 5′-AGATTGGTAGAAATTGCTGCAAAA-3′ and 5′-ACGCCACTTCCTTGCTCTTC-3′; PMP70, 5′-GGGAGAAGCAGACAATCCAC-3′ and 5′-CCGAAAGAAAATGAAATTATGTAGG-3′; Catalase, 5′-CCTTCAAGTTGGTTAATGCAGA-3′ and 5′-CAAGTTTTTGATGCCCTGGT-3′; PEX11a, 5′-TTCATCCGAGTCGCCAAC-3′ and 5′-CATGCATGCGTGCTGAGT-3′; CPT1b, 5′-GAGTGACTGGTGGGAAGAATATG-3′ and 5′-GCTGCTTGCACATTTGTGTT-3′; EHHADH, 5′-CCGGTCAATGCCATCAGT-3′ and 5′-CTAACCGTATGGTCCAAACTAGC-3′; DBP, 5′-GGGAGCAGTACTTGGAGCTG-3′ and 5′-TCAGCAATAACTGCTTCACATTTT-3′; MCAD, 5′-GCAACTGCCCGCAAGTTT-3′ and 5′-TACTCCCCGCTTTTGTCATATTC-3′; TNFα, 5′-CTGAGGTCAATCTGCCCAAGTAC-3′ and 5′-CTTCACAGAGCAATGACTCCAAAG-3′; IL-1β, 5′-TCTTTGAAGTTGACGGACCC-3′ and 5′-TGAGTGATACTGCCTGCCTG-3′; NOS2, 5′-GGCAGCCTGTGAGACCTTTG-3′ and 5′-GCATTGGAAGTGAAGCGTTTC-3′; COX2, 5′-GGGTTAAACTTCCAAAGGAGACATC-3′ and 5′-CAGCCTGGCAAGTCTTTAACCT-3′; IL6, 5′-TCGTGGAAATGAGAAAAGAGTTG-3′ and 5′-AGTGCATCATCGTTGTTCATACA-3′.

2.8. Statistical analysis

All values are represented as mean ± s.d. One-way analysis of variance was used to calculate the statistical significance of the results, and *P*-values <0.05 are considered statistically significant.

3. Results

3.1. Fenofibrate protects against liver damage caused by fasting–refeeding HFD

Fasting and refeeding diets are known to be associated with alterations in hepatic lipid and glucose metabolism which dramatically enhances expression of lipid metabolizing genes [19,20]. Fenofibrate, a peroxisome proliferator-activated receptor α (PPARα) agonist, is known to possess lipid-lowering effects and to decrease serum TG level in non-alcoholic fatty liver disease (NAFLD) [21–23]. Therefore, to determine the effects of fasting–refeeding high fat diet (HFD) and fenofibrate on hepatocytes, mice were fasted for 24 h and refed HFD for 16 h and also fenofibrate was administrated to both fed and refed mice. Body and liver weight of refed mice did not differ as of fed mice (data not shown). To evaluate the effects of refeeding HFD on hepatocytes, morphological analysis was performed in the liver section by H&E staining. Refed mice showed ballooning in liver (Fig. 1A indicated by arrow in refed mice). In contrast, the administration of fenofibrate in refed model mice showed no liver ballooning by H&E staining. Since liver ballooning is a marker for hepatocellular damage which ultimately leads to cell death [24,25], so terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed from the liver of the experimental mice. The number of TUNEL positive cells was increased in refed group as compared to fed control mice, while it was almost completely reduced with fenofibrate treatment (Fig. 1B and C). These data suggest that refeeding HFD after 24 h fasting may induce susceptibility to liver damage and apoptotic cell death, but fenofibrate administration may lessen the liver damage.

3.2. Fenofibrate ameliorates liver damage induced by fasting–refeeding HFD

Since mice are exposed to dietary fat after food deprivation, we speculated that a huge amount of free fatty acids are influx into liver, which leads to lipid accumulation in hepatocytes. To examine the accumulation of lipid, BODIPY staining was performed in the liver sections of experimental mice. BODIPY staining showed accumulation of lipid droplets in mice in the refed group, whereas fenofibrate administration inhibited the lipid accumulation (Fig. 2A). We also examined the levels of triglyceride (TG) extracted from the liver of experimental mice. Refed mice showed a 2-fold increase in liver TG levels as compared to the fed control mice. In contrast, TG levels in fenofibrate-treated refed mice were similar to the fed control group (Fig. 2B). Serum TG levels were also analyzed to examine the effect of fenofibrate, and mice injected with fenofibrate showed a reduced serum TG levels as compared to fed control or refed groups (Fig. 2C). These results indicate that refeeding HFD accumulates lipid which further enhances the liver TG level to induce liver damage, whereas fenofibrate may limit accumulation of lipid and TG and protect the liver from damage.

3.3. Fenofibrate blocks the activation of NF-κB and thus inhibits the activation of pro-inflammatory enzymes induced by refeeding HFD

Since inflammation contributes to the pathogenesis of metabolic disease [26,27], we evaluated the degree of inflammation in experimental mice by performing quantitative real-time PCR using cDNA prepared from total mRNA of the liver. The mRNA levels of NF-κB target genes, TNF-α, IL-1β, COX2 and NOS2, were

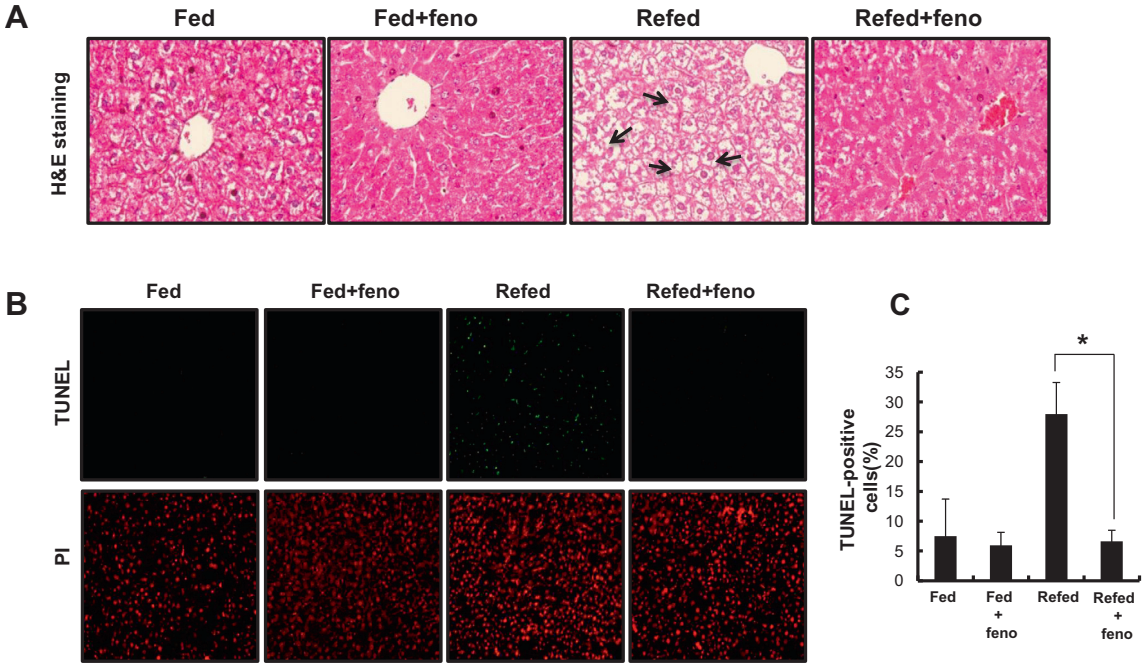

Fig. 1. Fenofibrate protects against liver damage caused by increased apoptosis due to fasting–refeeding HFD. (A) Mice were maintained in normal feeding (fed), normal feeding with fenofibrate injection (fed+feno), deprived of food for 24 h and refed with high fat diet (refed), or deprived of food for 24 h and refed with high fat diet with fenofibrate injection (refed+feno). Liver sections were subjected to H&E staining. The extent of ballooning degeneration (arrows) is indicated in refed mice. (B) TUNEL analysis of liver sections from mice treated as in (A). The TUNEL-positive nuclei were visualized as green. Nucleus is stained with propidium iodide (PI) as shown red. (C) Quantitation of TUNEL analysis. The values represent the mean ± S.D. ($n = 3$). *denotes $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

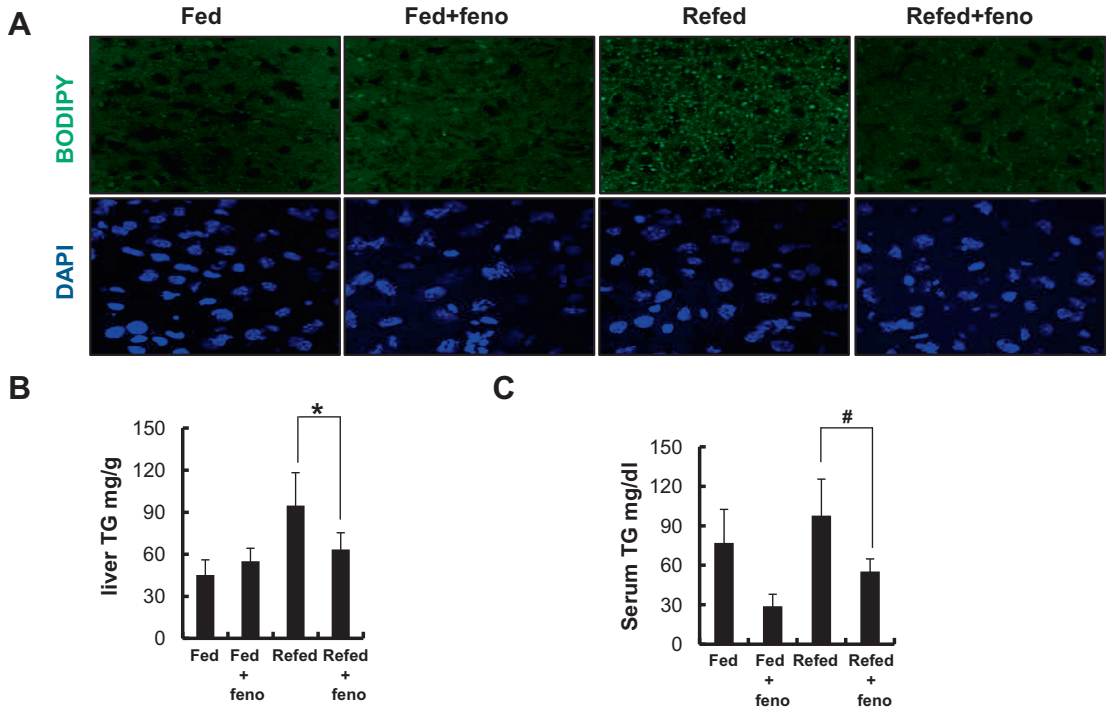

Fig. 2. Fenofibrate ameliorates the liver damage induced by fasting–refeeding HFD. (A) Mice were maintained in normal feeding (fed), normal feeding with fenofibrate injection (fed+feno), deprived of food for 24 h and refed with high fat diet (refed), or deprived of food for 24 h and refed with high fat diet with fenofibrate injection (refed+feno). Liver sections prepared from the mice were subject to BODIPY staining. The liver (B) and serum triglyceride (C) were measured from mice treated as in (A). The values represent the mean ± S.D. ($n = 4$). *denotes $p < 0.1$ and # indicates $p < 0.003$.

significantly induced by approximately 3.5-, 4-, 7- and 5-fold, respectively, in the refed group (Fig. 3A–D), whereas fenofibrate administration inhibited the activation of these genes. Furthermore, Immunohistochemistry (IHC) of paraffin-embedded liver sections was performed to examine the effect of NF-κB. IHC shows the translocation of NF-κB into the nucleus (Fig. 3E, arrow), and accordingly, NF-κB target enzymes TNF-α (Fig. 3F) and COX2 (Fig. 3G) were induced in the refed HFD group mice. In contrast, fenofibrate administration in refed group blocked the activation of NF-κB. These results suggest that refeeding HFD may induce inflammation which is almost completely inhibited by fenofibrate.

3.4. Fenofibrate induces peroxisomal and mitochondrial beta-oxidation enzymes

To examine the effect of fenofibrate on liver, expression of enzymes involved in peroxisomal beta-oxidation was measured. mRNA expression analyses showed that genes involved in peroxisomal beta oxidation and biogenesis, such as ACOX1, DBP, EHHADH and PEX11α, were greatly induced in fenofibrate-treated fed mice (Fig. 4A). In contrast, expression of these genes showed no differences in the refed group. Notably, fenofibrate administration in refed mice show a strong induction of these genes as compared to fed control mice. Immunoblot analysis also showed the induction of peroxisomal beta-oxidation protein (ACOX1, DBP) in both fenofibrate-treated groups, while there were no changes in refed mice (Fig. 4B). Catalase appeared to be expressed at similar levels independent of experimental conditions, suggesting that not all peroxisomal enzymes are regulated by fenofibrate treatment. In addition, the mRNA levels of mitochondrial matrix genes closely linked to mitochondrial beta oxidation, such as CPT1B and MCAD, were highly induced in both fenofibrate-treated, fed mice and refed fenofibrate-treated mice, while there were no differences between refed and fed control mice (Fig. 4C). These data strongly suggest that fenofibrate dramatically enhances peroxisomal beta-oxidation and proliferation in addition to mitochondrial beta-oxidation which protects liver function from the damage induced by fasting–refeeding HFD.

4. Discussion

In the current study, we used fenofibrate, a PPARα agonist, to examine the effect of PPARα activity on hepatic lipid metabolism in response to a fasting–refeeding HFD process that mimics a bad eating habit. By analyzing the liver tissues from mice subject to the fasting–refeeding process, we found that the fasting–refeeding HFD induces ballooning degradation of liver cells, hepatic triglyceride accumulation, and activation of NF-κB and its target genes. Therefore, a fasting–refeeding HFD process is sufficient to induce perturbation of liver function due to robust nutrient influx, especially lipid, which leads to triglyceride accumulation and stimulation of inflammatory response in hepatocytes to induce liver damage. Our results are consistent with a previous report in which inflammatory response is induced by fasting–refeeding with high-carbohydrate diet [28]. Conversely, short-term (2 days) treatment of fenofibrate protects the liver from refeeding-mediated injury, reduces hepatic triglyceride levels and induces the expression of genes involved in fatty acid oxidation and peroxisome proliferation.

PPARα is a nuclear hormone receptor and regulates lipid metabolism in the liver by altering transcription of many target genes related to fatty acid oxidation. Moreover, recent findings have shown that PPARα agonists improve hepatic steatosis in animal models, such as nonalcoholic fatty liver disease [25,29]. In this report, we show that enzymes for β-oxidation in two cellular organelles, the mitochondrion and peroxisome, are up-regulated by the PPARα agonist fenofibrate to activate fatty acid turnover.

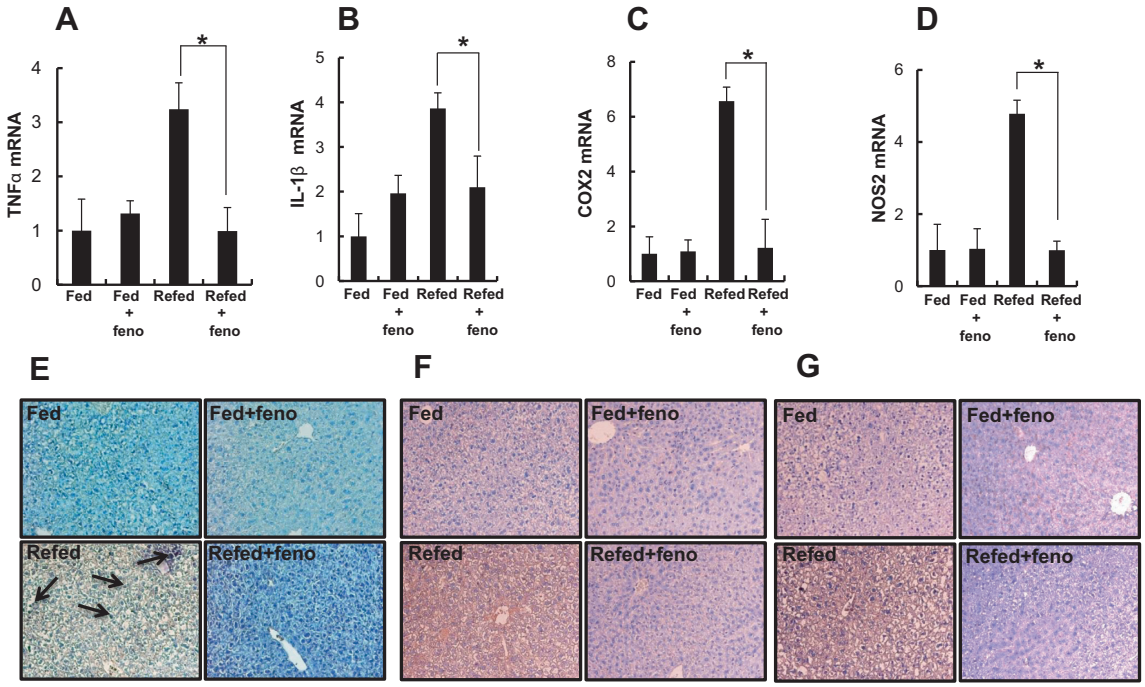

Fig. 3. Fenofibrate blocks translocation of NF-kB into the nucleus in refeeding HFD, and thus inhibits the activation of pro-inflammatory enzymes in refeeding HFD. Mice were maintained in normal feeding (fed), normal feeding with fenofibrate injection (fed+feno), deprived of food for 24 h and refed with high fat diet (refed), or deprived of food for 24 h and refed with high fat diet with fenofibrate injection (refed+feno). Total RNA isolated from the liver was subject to quantitative RT-PCR analysis of TNF-α (A), IL-1β (B), COX-2 (C), and NOS2 (D). The values represent the mean ± S.D. (n = 3–4). *indicates $p < 0.01$. Liver sections prepared from the mice treated as in (A) were stained with NF-κB (E), TNF-α (F), or COX2 (G) antibody for immunohistochemistry.

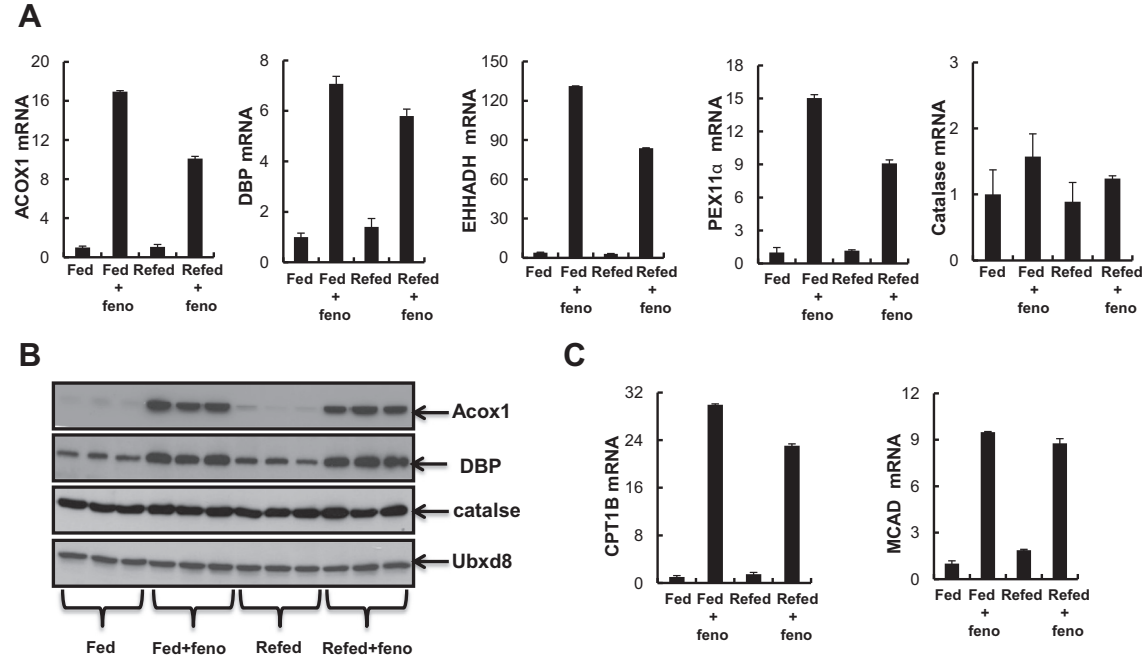

Fig. 4. Effects of fenofibrate treatment on peroxisomal and mitochondrial beta oxidation enzymes. (A) Mice were maintained in normal feeding (fed), normal feeding with fenofibrate injection (fed+feno), deprived of food for 24 h and refed with high fat diet (refed), or deprived of food for 24 h and refed with high fat diet with fenofibrate injection (refed+feno). Total RNA isolated from the liver was subject to quantitative RT-PCR analysis of ACOX1, DBP, EHHADH, PEX11α and Catalase gene. (B) Immunoblot analysis of ACOX1, DBP, or catalase from liver homogenates of mice treated as in (A). (C) Total RNA isolated from the liver treated as in (A) was subject to quantitative RT-PCR analysis for CPT1B and MCAD.

For example, expression of enzymes involved in peroxisomal fatty acid oxidation, such as ACOX1, DBP, and EHHADH, and the proteins in peroxisome proliferation, such as Pex11a, are enhanced by fenofibrate treatment. In addition, genes related to mitochondria fatty acid oxidation, such as CPT1B and MCAD, are also upregulated by fenofibrate. Therefore, activation of PPARα by treating fenofibrate prevents the intrahepatic lipid storage by increasing mitochondrial and peroxisomal fatty acid oxidation and attenuates

accumulation of the toxic lipid species in response to fasting–refeeding with high fat diet.

Since ACOX1, the enzyme responsible for the first step of peroxisomal fatty acid oxidation, is a FAD-dependent oxidase and its expression is highly enhanced by fenofibrate treatment, this enzyme transfer electrons from fatty acyl-CoA to oxygen, which in turn generates H_2O_2 and enoyl-CoA [30]. As the activity of ACOX1 is increased, hydrogen peroxide is accumulated to induce oxidative stress. Peroxisome contains several anti-oxidant enzymes to decompose of reactive oxygen species [31]. Catalase, one of the most abundant enzyme in peroxisome, is known to eliminate hydrogen peroxide generated from the peroxisomal fatty acid oxidation process [31,32]. Unexpectedly, expression of catalase is not affected by fenofibrate even in the condition that peroxisomal fatty acid oxidation is predominated and H_2O_2 generation is enhanced. These results implicate that catalase may not be involved in alleviating oxidative stress produced by fatty acid oxidation in peroxisomes. Therefore, the functional role of catalase in liver lipid metabolism needs to be elucidated.

Taken together, our findings indicate that hepatic lipid metabolism is drastically changed by nutrient availability and that irregular eating behavior, such as skipping meals, may result in metabolic dysfunction in liver. Our result also suggests that fenofibrate, a PPARα agonist, may improve liver abnormalities induced by fasting–refeeding.

Disclosure

All the authors declared no competing interests.

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