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The selective PPARα modulator K-877 efficiently activates the PPARα pathway and improves lipid metabolism in mice

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Author Contributions

Y.N. and H. Shimano designed the experiments and wrote the manuscript. K.T., S-I.H., Y.M., A.S., F.O., H.O., Y.O., T.M., and Y.N. performed the experiments. M.S., H.I., S.Y., N. Yahagi, and H. Suzuki were involved in project planning. N. Yamada supervised this study and contributed crucial ideas to the project.

Abstract

Aims/Introduction: Peroxisome proliferator-activated receptor α (PPARα) is a therapeutic target for hyperlipidemia. K-877 is a new selective PPARα modulator (SPPARMα) that activates PPARα transcriptional activity. The aim of the present study was to assess the effects of K-877 on lipid metabolism in vitro and in vivo compared with those of classical PPARα agonists.

Materials and Methods: To compare the effects of K-877 on PPARα transcriptional activity with those of the classical PPARα agonists Wy14643 (Wy) and fenofibrate (Feno), the cell-based PPARα transactivation luciferase assay was performed. WT and *Ppara*$^{-/-}$ mice were fed with a moderate fat diet (MF) for 6 days and methionine–choline deficient diet (MCD) for 4 weeks containing Feno or K-877.

Results: In luciferase assays, K-877 activated PPARα transcriptional activity more efficiently than the classical PPARα agonists fenofibrate (Feno) and Wy14643 (Wy). After feeding on a normal diet containing 0.001% K-877 or 0.2% Feno for 6 days, mice in the K-877 group exhibited significant increases in the expression of *Ppara* and its target genes, leading to marked reductions in plasma triglyceride levels compared with those observed in Feno treated animals. These K-877 effects were blunted in *Ppara*$^{-/-}$ mice, confirming that K-877 activates PPARα. In further experiments, K-877 (0.00025%) and Feno (0.1%) equally improved the pathology of MCD-induced nonalcoholic fatty liver disease, with increased expression of hepatic fatty acid oxidation genes.

Conclusions: The present data indicate that K-877 is an attractive PPARα modulating drug and can

efficiently reduce plasma triglyceride levels, thereby alleviating the dysregulation of lipid metabolism.

Keywords

SPPARMα, PPARα, lipid metabolism

Introduction

Impaired nutrient homeostasis is a common characteristic of metabolic disorders such as obesity, diabetes, cardiovascular diseases, and fatty liver disease. Nutrient homeostasis is tightly regulated via the balance between energy producing pathways, such as ketogenesis, gluconeogenesis, and lipid synthesis, and energy utilization pathways, such as lipid oxidation. Because of the emerging epidemic of obesity and diabetes, the factors determining progression of nonalcoholic fatty liver disease (NAFLD) present a major clinical challenge. In particular, fatty liver can progress to nonalcoholic steatohepatitis (NASH) following overwhelming of the adaptive mechanisms that mediate lipid partitioning and metabolism and protect hepatocytes from lipotoxicity of excess fatty acids (FAs) and other lipids, resulting in inflammation and fibrosis.

Peroxisome proliferative-activated receptors (PPARs) are members of the nuclear receptor superfamily and include PPARα, PPARβ/δ, and PPARγ. Upon ligand binding, PPARs form heterodimers with the retinoid X receptor and interact with PPAR response elements to regulate target gene expression. PPARα is most prominently expressed in the liver and is activated by

hypolipidemic fibrate-class drugs (fibrates). PPARα controls lipid flux in the liver by modulating fatty acid transport and β-oxidation and improves plasma lipid profiles by decreasing triglyceride (TG) levels and increasing high-density lipoprotein (HDL) cholesterol levels. In addition, PPARα activation inhibits inflammatory genes that are induced by nuclear factor-κB and decreases the expression of acute-phase response genes. Accordingly, PPARα deficiency increases susceptibility to NAFLD, NASH, hepatic inflammation, and acute phase responses [1]. Fibrates such as gemfibrozil, bezafibrate, and Feno decrease plasma TG levels and increase HDL-cholesterol levels in patients with hyperlipidemia and Type-2 diabetes and can prevent coronary heart disease and stroke [2-6]. However, these drugs are weak agonists of PPARα, have poor substrate selectivity, and require high clinical doses. Therefore, a potent and selective PPARα agonist is needed for patients with metabolic syndrome. K-877 is a novel selective PPARα modulator (SPPARMα) that enhances PPARα activity [7]; it also elicits higher PPARα activation than other fibrates, with lower EC_{50} values and higher PPAR subtype selectivity [8].

In this study, we compared the effects of K-877 on lipid metabolism and hepatic gene expression related to NASH/NAFLD with the classical PPARα agonists, fenofibrate and Wy14643 in vitro and in vivo.

Materials and Methods

Reagents

K-877 was kindly provided by Kowa Co. Ltd. Fenofibrate and Wy14643 were purchased from Sigma-Aldrich.

Animals

Eight-week-old male C57BL/6J (wild-type, WT) mice were obtained from CLEA Japan. B6;129S4-*Ppara*$^{tm1Gonz/J}$ (*Ppara*$^{-/-}$) mice were purchased from the Jackson Laboratory. MCD-diet analyses were performed on 8-week-old male mice that were fed for 4 weeks and sacrificed without fasting. All animal husbandry procedures and experiments were compliant with the University of Tsukuba's Regulations for Animal Experiments and were approved by the Animal Experiment Committee at the University of Tsukuba.

Histological analysis

Harvested livers were fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE).

Plasmids

The expression vector for the Gal4-PPARα (pM Gal4-PPARα) fusion protein was generated by inserting a human PPARα fragment (166–468 aa) downstream of Gal4 in the pM vector (Clontech). The GAL4 UAS–LUC vector contains eight copies of the UAS Gal4-binding site [9].

Cell culture

Mouse AML12.2 hepatoma cells were maintained in Dulbecco's Modified Eagle Medium/Ham's F12 media supplemented with ITS Liquid Media Supplement (SIGMA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). Cells were incubated with Feno (50 μM), or K-877 (5 and 50 μM) for 48 h.

Transfections and luc assays

HepG2 cells were grown at 37°C in an atmosphere of 5% CO_2 in DMEM containing 25 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). Transfection studies were performed in cells on 24-well plates. Cells were transfected with pM Gal4-PPARα DBD and GAL4 UAS luciferase vectors [9], and a pRL-SV40 plasmid as a reference (Promega) using X-tremeGENE 9 (Roche). After 24 h of transfection, Wy (50 μM), Feno (30 μM), or K-877 (50 nM) were added to the medium. After additional 24-h incubation, firefly luciferase activity was measured and normalized to that of *Renilla*.

Metabolic measurements

Plasma levels of TG, non-esterified fatty acid (NEFA), total cholesterol (TC), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and liver TG and TC levels were measured as described previously [10].

Analysis of gene expression

Total RNA from cells and tissues was prepared using a Trizol reagent (Invitrogen). Prior to real-time PCR analyses, total RNA was reversed transcribed into cDNA using a reverse transcriptase according to the manufacturer's instructions (Invitrogen). Real-time PCR was performed using an ABI Prism 7300 system (ABI) with TaqMan probes (Invitrogen) and a SYBR Green Master Mix (Roche)[11]. Primer sequences are available upon request.

Statistical analyses

Comparisons of treatment groups were made using Tukey-Kramer post-hoc tests, and differences were considered significant when $p < 0.05$. All data are expressed as means ± standard errors of the mean (SEM).

Results

K-877 effectively activates PPARα transcriptional activity

To compare the effects of K-877 on PPARα transcriptional activity with those of the classical PPARα agonists Wy and Feno, cell-based transactivation assays were performed using a Gal4-luciferase assay system. After co-transfecting human HepG2 hepatoma cells with pM-GAL4 PPARα ligand-binding domain (LBD) and GAL4 UAS reporter vectors, cells were treated with K-877 for 24 h. Although K-877, Wy, and Feno induced PPARα luciferase activation in dose-dependent manner, the dose-response curve of K-877 was shifted to left side compared with those of Wy14643 and Feno (Fig. 1a). K-877 is a highly potent agonist of PPARα transcriptional activity with half-maximal effective concentrations (EC50) of 0.49 nM. The PPARα agonists Wy (50 μM), Feno (30 μM), or K-877 (50 nM) were treated for 24 h. PPARα luciferase activation by K-877 was comparable to that by Wy or Feno (Fig. 1b). Considering the relative concentrations of these drugs, results indicate that K-877 activates the PPARα transcription activity more effectively than the other agents. To determine the effects of K-877 on fibroblast growth factor 21 (*Fgf21*) expression, a typical target gene of PPARα, in vitro, mouse AML12.2 hepatoma cells were incubated with the PPARα agonists Feno (50 μM) or K-877 (5 and 50 μM) for 48 h based on a previous report [12]. Both doses of K-877 significantly increased *Fgf21* expression, but increase in expression by Feno was slight (Fig. 1c). Thus, K-877 can efficiently activate *Fgf21* expression.

K-877 decreased plasma lipid levels in WT mice

Based on a previous report [7], 8-week-old male WT mice were fed a MF diet containing 0.2% (w/w) Feno or 0.001% (w/w) K-877 for 6 days. Although the dose of K-877 was 200-fold lower than that of Feno, compared with no treatment, K-877 and Feno significantly reduced plasma TG levels, and tended to reduce TC, and NEFA levels (Fig. 2a), and the effects of both agonists were blunted in *Ppara*$^{-/-}$ mice (Fig. 2a). These data indicate that considering the concentrations of agonists, K-877 elicits greater PPARα-mediated lipid-lowering effects than Feno. Accordingly, hepatic gene expression of *Ppara* and its target genes, such as those encoding cAMP responsive element binding protein 3-like 3 (*Creb3l3*), *Fgf21*, acyl-CoA oxidase 1, palmitoyl (*Acox1*), and carnitine palmitoyltransferase 1a, liver (*Cpt1a*), were significantly increased by both PPARα agonists (Fig. 2b). These changes in gene expression were similar in the presence of K-877 and Feno in spite of dose differences, indicating that K-877 has more powerful effects on PPARα activation than Feno. In addition, the effects of both agonists were abolished in *Ppara*$^{-/-}$ mice, confirming that these agonists target the PPARα pathway.

K-877 suppresses MCD-induced liver injury in normal mice, but not in *Ppara*$^{-/-}$ mice

To compare the effects of PPARα agonists on the progression of NAFLD, WT and *Ppara*$^{-/-}$ mice were fed an MCD diet containing 0.1% Feno or 0.00025% K-877 for 4 weeks, and their optimum doses were determined according to previously reported methods [7, 12]. This diet has

been used extensively to produce diet-induced animal models of NASH that exhibit similar histology to that of human NASH [1]. Histological analyses of HE stained liver sections from WT mice showed that the MCD diet led to slight lipid accumulation in hepatocytes (Fig. 3a). The addition of Feno and K-877 suppressed MCD-induced lipid accumulation (Fig. 3a). Moreover, sections from MCD-fed *Ppara*$^{-/-}$ mice exhibited greater lipid accumulation and macrophage invasion than those from MCD-fed WT mice, but the addition of Feno and K-877 could not improve them (Fig. 3a). Hepatic TG and TC contents were significantly increased in MCD diet-fed mice than in MF diet-fed mice (Fig. 3b). The addition of K-877 and Feno into MCD diets tended to reduce liver TG land TC levels, and *Ppara*$^{-/-}$ mice exhibited more severe liver lipid accumulation than WT mice (Fig. 3b). However, PPARα agonists did not improve these phenotypes significantly (Fig. 3b). In further analyses, increased plasma ALT and AST levels were observed in MCD diet-fed WT mice, compared with those in MF diet-fed WT mice (Fig. 3c). The administration of K-877 or Feno tended to decrease MCD diet-induced ALT and AST levels. However, both agonists failed to suppress these increases in *Ppara*$^{-/-}$ mice (Fig. 3c). Taken together, these data suggest that K-877 and Feno ameliorate MCD diet-induced fatty liver progression.

K-877 activates PPARα target gene expression and reduces *Xbp1s* expression in the liver of MCD-fed mice

Hepatic gene expression was determined in WT and *Ppara*$^{-/-}$ mice after feeding on a MCD diet containing PPARα agonists for 4 weeks. Consistent with the expression levels of *Ppara* and its target genes *Creb3l3* in normal mice, *Acox1* and *Cpt1a* were significantly increased to similar levels in the presence of PPARα agonists (Fig. 4). However, *Fgf21* expression was decreased in the presence of PPARα agonists. Moreover, no apparent differences in inflammatory and macrophage hepatic gene expression were observed between MCD diet-fed WT mouse groups. The administration of PPARα agonists on *Ppara*$^{-/-}$ mice showed increased *Cd68* expression, supporting the increase of macrophages including Kupffer cells. However, the MCD diet induced endoplasmic reticulum stress (ER stress) markers such as X-box binding protein 1s (*Xbp1s*) in WT mice. In addition, the effects of K-877 on *Xbp1s* expression were abolished in in *Ppara*$^{-/-}$ mice. In subsequent experiments, K-877 suppressed MCD diet-induced *Xbp1s* expression more efficiently than Feno, thereby ameliorating ER stress in MCD diet-fed WT mice. In contrast, neither PPARα agonist suppressed ER stress-related gene expression in *Ppara*$^{-/-}$ mice. These data indicate that K-877 increases PPARα target genes that are related to fatty acid oxidation and reduces *Xbp1s* expression, leading to decreased liver injury.

Discussion

In this study, we showed that K-877 specifically and efficiently activates PPARα transactivation activity in vitro and in vivo. Moreover, K-877 had greater lipid-lowering effects than the classical PPARα agonists in mice and significantly induced PPARα target genes. These effects reduced MCD-induced liver injury by increasing PPARα target gene expression and decreasing ER stress in the liver. Therefore, K-877 may be an effective drug for hyperlipidemia.

Fibrates are widely used to ameliorate the macro- and microvascular risks associated with metabolic syndrome. However, these agents are weak PPARα agonists and have limited efficacy due to dose-related adverse events. To address this problem, a new generation of PPARα-specific agonists, known as SPPARMαs, has been developed to maximize receptor-mediated effects and diminish side effects. In luciferase analyses, we confirmed that K-877 activates PPARα at 1000-fold lower doses than Feno and Wy. K-877 increases *Fgf21* expression, one of the typical target genes for PPARα in AML12.2 cells. FGF21 is known to ameliorate obesity, diabetes, and hyperlipidaemia by inducing lipid catabolism [13, 14]. The in vivo studies showed that K-877 and Feno induced the PPARα target genes *Fgf21*, *Acox1*, and *Cpt1a* in the liver of MF-fed WT mice, suggesting that these compounds activate hepatic fatty acid oxidation. These effects were similar in the presence of low doses of K-877, or high doses of Feno, suggesting superiority of K-877 as a clinical agent. As a results, the administration of K-877 and Feno in MF-fed WT mice reduced plasma lipids including TG, TC and NEFA. Previous reports have shown that some PPARα agonists prevent the progression

of NAFLD [15, 16]. Accordingly, K-877 efficiently increased the PPARα transcriptional activity and subsequently transactivated fatty acid oxidation genes, including *Acox1* and *Cpt1a*, resulting in reduced hepatic lipid accumulation in MCD-fed mice. However, *Fgf21* expression decreased in MCD-fed WT mice in the presence of PPARα agonist compared with that in MCD-fed WT mice without agonists. *Fgf21* expression was induced in both WT and *Ppara*$^{-/-}$ mice in response to MCD, suggesting that a PPARα independent mechanism underlies MCD-induced *Fgf21* expression.

ER stress is associated with accumulation of misfolded and unfolded proteins in the ER lumen. ER plays an essential role in controlling lipid metabolism. XBP1s induces the expression of multiple inflammatory cytokines [17], suggesting important roles of ER stress in the pathology of NAFLD. K-877 reduced hepatic *Xbp1s* expression, indicating potential roles in the management of ER stress. Moreover, reduced lipid accumulation in the presence of K-877 might lead to decreases in the ER stress-related gene expression. Lastly, the effects of K-877 in *Ppara*$^{-/-}$ mice were blunted, confirming that this agonist specifically targets PPARα.

Selective activation of PPARα by K-877 was associated with beneficial changes in liver disease markers, suggesting the potential of this novel agent in the treatment of NASH/NAFLD may relate to PPARα pathway activation and reduced ER stress. However, the mechanisms of K-877-mediated PPARα activation remain unknown, warranting further studies to characterize the specificity of K-877 for PPARα activation, recruitment of cofactors, and downstream gene expression.

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Figure Legends

Figure 1. K-877 activates the PPARα transcriptional activity.

(a, b) HepG2 cells were co-transfected with GAL4 UAS-LUC and pM GAL4-hPPARα LBD vectors, and with a pRL-SV40 plasmid as a reference in 24-well plates for 24 h. Cells were then treated with Wy, Feno, or K-877 for 48 h and were harvested at 48 h after transfection. Luciferase activity was measured and normalized to that of *Renilla* luciferase activity. (a) Dose–response curves of PPARα transactivation. (b) Cells were then treated with Wy (50 μM), Feno (30 μM), or K-877 (50 nM). n = 8 per group. (c) AML12.2 cells were treated with Feno (50 μM) or K-877 (5 and 50 μM) for 48 h. *Fgf21* expression was determined by QPCR. n = 9 per group; *$p < 0.05$ and **$p < 0.01$.

Figure 2. K-877 reduces plasma lipid levels and increases the expression of hepatic fatty acid oxidation genes in wild type, but not in *Ppara*$^{-/-}$ mice.

Eight-week old male wild type and *Ppara*$^{-/-}$ mice were administrated with Feno (0.2%) or K-877 (0.001%) for 6 days. Plasma TG, TC levels (a), and hepatic gene expression (b) in WT and *Ppara*$^{-/-}$ mice; n = 9–13 per group; *$p < 0.05$ and **$p < 0.01$.

Figure 3. K-877 suppresses MCD diet-induced NAFLD in wild type, but not in *Ppara*$^{-/-}$ mice.

Eight-week-old male wild type and *Ppara*$^{-/-}$ mice were fed MF or MCD diets containing Feno (0.1%) or K-877 (0.00025%) for 4 weeks. HE staining in the liver (a), hepatic lipid contents (b), and plasma

AST and ALT levels (c) were determined in WT and *Ppara*$^{-/-}$ mice; n = 5–10 per group; *p < 0.05 and **p < 0.01.

Figure 4. Hepatic gene expression in wild type and *Ppara*$^{-/-}$ mice fed the MCD diet with K-877 or Feno for 4 weeks.

Eight-week-old male wild type and *Ppara*$^{-/-}$ mice were fed MF or MCD diets containing Feno (0.1%) or K-877 (0.00025%) for 4 weeks. Hepatic gene expression profiles of WT and *Ppara*$^{-/-}$ mice; n = 5–10 per group; *p < 0.05 and **p < 0.01.

Fig. 1 Takei

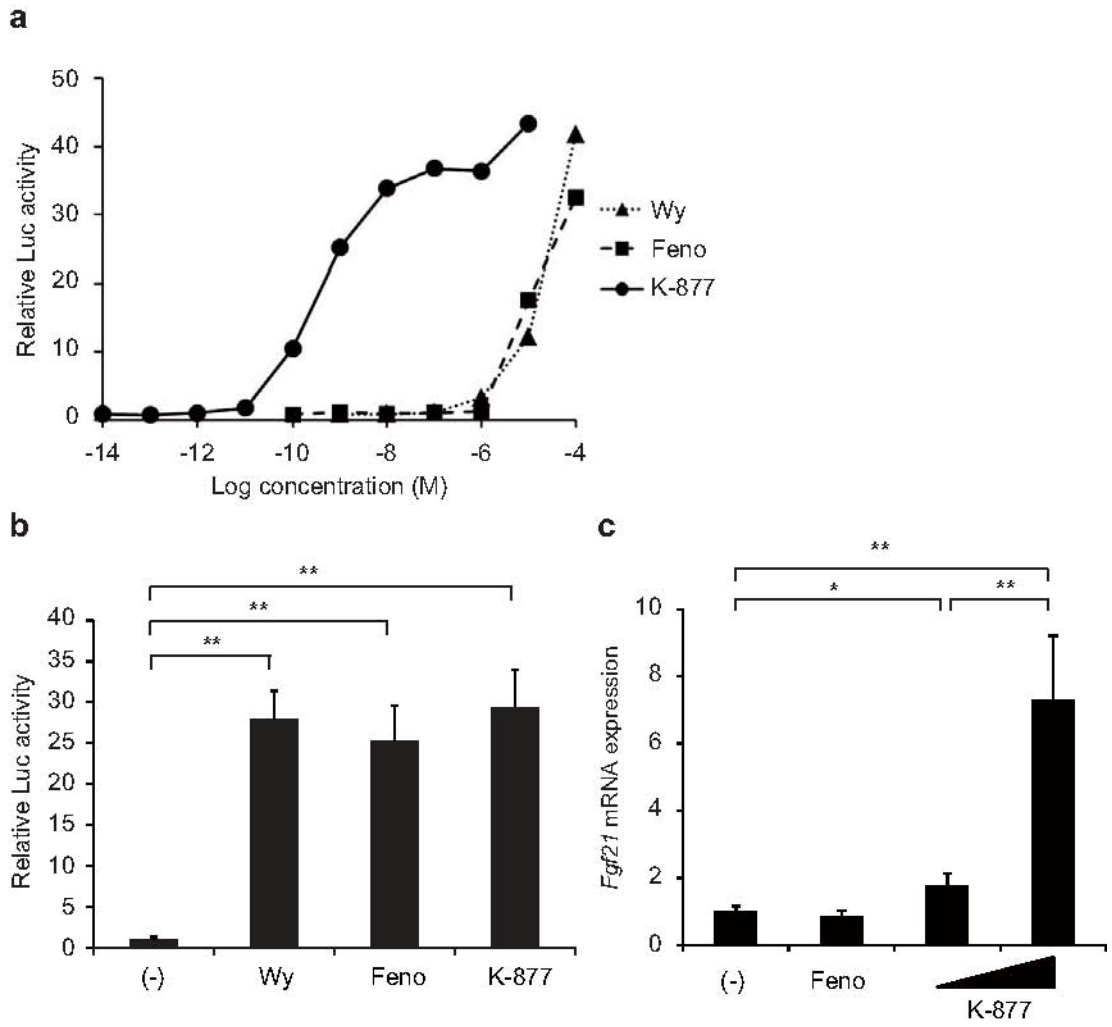

Fig. 2

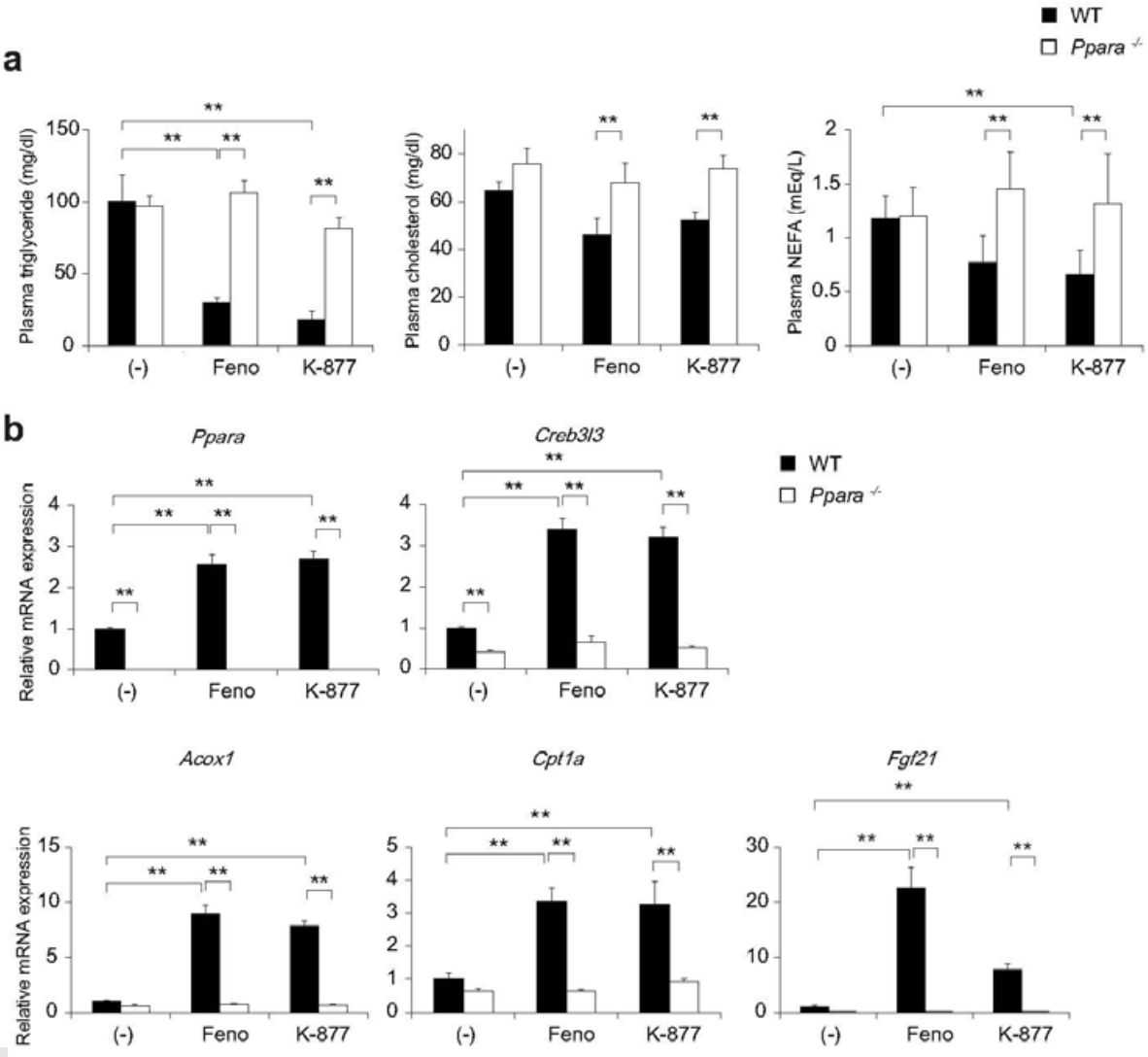

Fig. 3

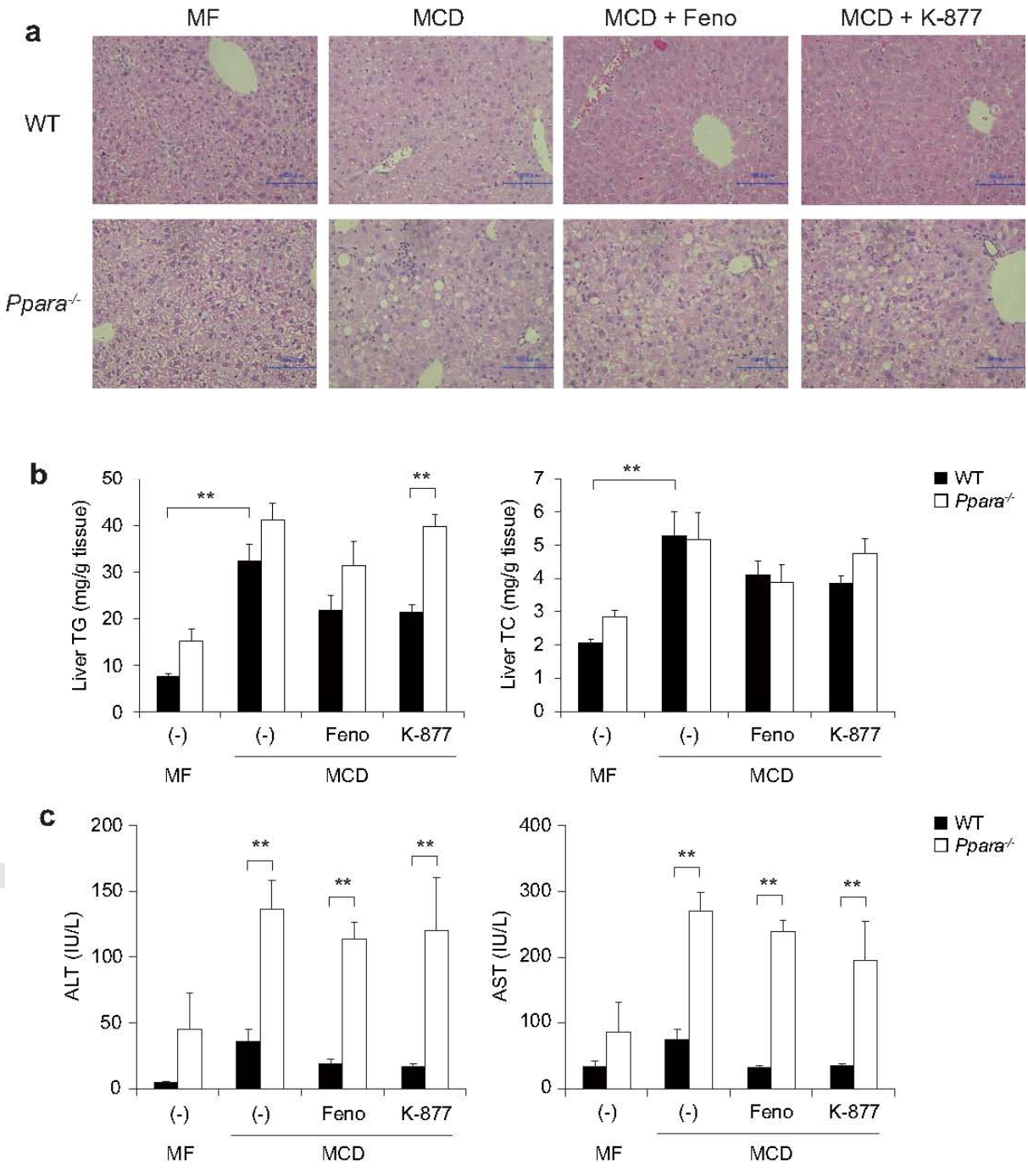

Fig. 4