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The Role of FXR and TGR5 in Reversing and Preventing Progression of Western Diet-induced Hepatic Steatosis, Inflammation, and Fibrosis in Mice

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Abstract

Nonalcoholic steatohepatitis (NASH) is the most common chronic liver disease in the US, partly due to the increasing incidence of metabolic syndrome, obesity, and type 2 diabetes. The roles of bile acids and their receptors such as the nuclear receptor farnesoid X receptor (FXR) and the G protein-coupled receptor TGR5, on the development of NASH are not fully clear. C57BL/6J male mice fed a Western diet (WD) develop characteristics of NASH, allowing determination of the effects of FXR and TGR5 agonists on this disease. Here we show that the FXR-TGR5 dual agonist INT-767 prevents progression of WD-induced hepatic steatosis, inflammation, and fibrosis, as determined by histological and biochemical assays and novel label-free microscopy imaging techniques, including Third Harmonic Generation, Second Harmonic Generation, and Fluorescence Lifetime Imaging Microscopy. Furthermore, we show INT-767 decreases liver fatty acid synthesis and fatty acid and cholesterol uptake, as well as liver inflammation. INT-767 markedly changed bile acid composition in the liver and intestine, leading to notable decreases in the hydrophobicity index of bile acids, known to limit cholesterol and lipid absorption. In addition, INT-767 upregulated expression of liver p-AMPK, SIRT1, PGC-1α, and SIRT3, which are master regulators of mitochondrial function. Finally, we found INT-767 treatment reduced WD-induced dysbiosis of gut microbiota. Interestingly, the effects of INT-767 in attenuating NASH were absent in FXR-null mice, but still present in TGR5-null mice. Our findings support treatment and prevention protocols with the dual FXR-TGR5 agonist INT-767 arrest
progression of WD-induced NASH in mice mediated by FXR-dependent, TGR5-independent mechanisms.
Keywords: NASH, FXR-TGR5, Fibrosis, Lipid Metabolism, Inflammation, Bile acid
Abbreviations: NASH, nonalcoholic steatohepatitis; NAFLD, nonalcoholic fatty liver disease; WD, Western diet; FXR, farnesoid X receptor; THG, third harmonic generation; SHG, second harmonic generation; FLIM, fluorescence lifetime imaging microscopy; LF, low-fat control diet; PLIN2, perilipin-2; MCD, methionine and choline deficient diet.
1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of liver disease in the United States, in part due to the increasing incidences of metabolic syndrome, obesity, and type 2 diabetes mellitus (1). About 20 percent of people with NAFLD develop NASH (2). The histological phenotype of NASH extends from steatosis to steatohepatitis, which can then progress to advanced fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (1,3). Recent human biopsy studies indicate that the transition from steatosis to steatohepatitis is a dynamic process, and the presence of fibrosis is the greatest prognostic value to predict the progression of NASH to cirrhosis and HCC (4).

Current clinical management of NASH is based on lifestyle intervention, including calorie-restricted diets and increased physical activity to promote weight loss (5). However, since weight loss is not always successful, additional surgical (6) and pharmacological (7) interventions are being explored. Bariatric surgery, including Roux-en-Y gastric bypass and vertical sleeve gastrectomy, were shown to result in significant weight loss and improvement of NASH (8). Several pharmacological interventions, including vitamin E, thiazolidinediones (TZDs or PPAR-γ agonists), and the FXR agonist obeticholic acid (OCA, 6α-ethyl-3α, 7α-dihydroxy-5β-cholan-24-oic acid, INT-747) have shown beneficial effects in NASH, but no drug is currently approved for this indication (9-12). Importantly, OCA was also shown to improve fibrosis in NASH patients (12) and has recently completed the interim analysis of a pivotal phase 3 trial in NASH (12).
Interestingly, vertical sleeve gastrectomy results in significant alterations in bile acid metabolism and bile acid levels. For example, increases in serum cholic acid (CA) and tauroursodeoxycholic acid (TUDCA) were found that could explain the positive effects of bile acid receptor activation (13). In mouse models of obesity, the beneficial effects of vertical sleeve gastrectomy were first shown to be FXR-dependent (14), and later also determined to be TGR5-dependent (15).

TGR5 is the G-protein-coupled bile acid receptor 1 (GPBAR1); a member of the rhodopsin-like subfamily of GPCRs. TGR5 is activated by bile acids, with the secondary bile acid lithocholic acid (LCA) and its tauro-conjugate (T-LCA) being the most potent endogenous agonists (16-18). The potent and selective TGR5 agonist (6α-ethyl-23(S)-methyl-3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid; also known as INT-777 was shown to blunt diet-induced obesity and fatty liver (19) and was thereby hypothesized to play a role in preventing NASH.

We previously characterized the semi-synthetic bile acid derivative INT-767 (6α-ethyl-3α, 7α, 23-trihydroxy-24-nor-5β-cholan-23 sulfate sodium salt) as the first agonist able to potently and selectively activate both FXR and TGR5 (20). INT-767 has 3 times higher potency than OCA to activate FXR, which makes it possible to deliver better efficacy at the same dose. Indeed, recently it has been reported that INT-767 can prevent and reverse diet-induced metabolic disorders such as NAFLD and atherosclerosis (21-27). In this study, we aim to further define the impact of INT-767 on
the reversal and/or arrest of progression of Western Diet (WD)-induced NASH, which is an increasingly relevant model to human disease.

The results indicate that the dual FXR-TGR5 agonist INT-767 can improve WD-induced liver injury and prevent progression to NASH. The effects of INT-767 reversing steatosis and fibrosis were mediated by FXR-dependent mechanisms, as shown by lack of efficacy in WD-fed Fxr-null mice, but not in Tgr5-null mice.
2. Results

2.1. INT-767 regulates expression of FXR and TGR5 target genes in liver and intestine: WD feeding did not significantly alter mRNA abundances of FXR target gene mRNAs Cyp7a1, Cyp8b1, Shp, or Bsep in mouse liver. As expected, treatment with the dual FXR/TGR5 agonist INT-767 markedly decreased Cyp7a1 and Cyp8b1 mRNA levels, while increasing Shp and Bsep mRNA levels, compared to non-treated controls (Figure 1A). These results agree with the known ability of FXR activation to decrease hepatic bile acid levels by stimulating SHP-dependent suppression of CYP7A1-mediated bile acid synthesis, and by increasing BSEP-dependent bile acid export (28,29). In the ileum, INT-767 increased FXR targets Fgf15 and Shp mRNAs (Figure 1B), and Gcg mRNA encoded by a TGR5 target gene relative to untreated WD-fed mice (Figure 1C). These results are in agreement with the known actions of FXR and TGR5 mediated signaling in the ileum (19,28,29). However, Tgr5 mRNA levels did not change after INT-767 treatment (data not shown).

2.2. INT-767 treatment improves WD-induced NASH: WD-fed mice developed enlarged livers and showed liver injury with marked hepatocellular steatosis, liver inflammation, and fibrosis, typical of NASH (30). We undertook studies to determine whether INT-767 treatment could improve established liver pathology in WD-fed mice. Accordingly, following WD feeding for 3 months to allow induction of steatosis and hepatocellular liver injury (Figure 2A), we added INT-767 to the WD and continued the experiment for another 3 months. Mice fed WD alone for 6 months had increased body
and liver weights. Treatment with INT-767 for the last 3 months of the experimental period reversed the liver weight increase, without affecting body weight, food intake, or glucose homeostasis (Table 1). INT-767 also decreased serum AST and ALT levels. While INT-767 did not alter fasting serum triglycerides, it lowered significantly the WD-induced increase in serum cholesterol.

2.3. Treatment of WD-fed mice with INT-767 improves liver triglyceride and cholesterol ester accumulation: Feeding mice the WD for 3 months resulted in steatosis with the start of inflammation (Figure 2A). Treatment with INT-767 during the following 3 months reversed the WD-induced steatosis as determined by H&E staining (Figure 2B) and perilipin-2 (PLIN2) immunohistochemistry (Figure 2C). The reversal of liver steatosis was also determined by fluorescence lifetime imaging microscopy (FLIM) and THG (Figure 2D). FLIM detected an increase in oxidized lipid species induced in WD-fed mice, which was prevented by INT-767 (Figure 2D). INT-767 intervention significantly reduced the size of lipid droplets in WD-fed mice as revealed by THG histogram (Figure 2D). This was revealed by the zoomed-in (90 µm) images showing small droplets and from the histogram (Figure 2D).

Gas chromatography-mass spectroscopy analyses were used to further define how INT-767 intervention reversed hepatocellular steatosis. INT-767 intervention reduced liver triglycerides in WD-fed mice to control levels (Figure 2E). The reduction in triglyceride levels was associated with significant alterations in hepatic triglyceride fatty acid composition (Supplementary Figure 1A), which resulted in a reduction in the fatty
acid desaturation indexes (Supplementary Figure 1B), decreased ratios of unsaturated to saturated fatty acids (Supplementary Figure 1C), and increased polyunsaturated to monounsaturated fatty acids (Supplementary Figure 1D). Detailed analysis of lipogenic pathways in liver tissue demonstrated that INT-767 intervention significantly reduced mRNA levels of genes involved in regulation of de novo lipogenesis (SREBP-1c, ChREBP-α, and ChREBP-β), fatty acid desaturation (SCD1), and fatty acid uptake (CD36 and FABP1) in both LF and WD-fed mice (Figure 2F, G).

INT-767 intervention also reversed the WD-induced increases in hepatic cholesterol ester levels (Figure 2H). This reversal was associated with significantly decreased expression of the oxidized LDL receptor, LOX-1 (Figure 2I), which, together with CD36 (Figure 2G), is a major mediator of cholesterol uptake. Notably, INT-767-mediated suppression of hepatic cholesterol levels subsequently upregulated the SREBP-2 pathway and downregulated LXR signaling as shown by increased SREBP-2-mediated cholesterol synthesis and decreased LXR-mediated expression of cholesterol efflux genes (Figure 2J).

2.4. INT-767 improves WD-induced liver inflammation: Livers of mice fed WD exhibited evidence of inflammation as demonstrated by H&E staining (Supplementary Table 1), CD3 immunohistochemistry (Figure 3A), and increased mRNA levels of MCP-1, TNFα, and IL-1β (Figure 3B). Treatment of WD-fed mice with INT-767 improved all of these inflammatory alterations.
The mouse liver injury scoring system (31) revealed that the WD drastically increased the percentage of hepatocytes showing macrosteatosis compared to those fed LF diet (Supplementary Table 1). No mice fed the WD and treated with INT-767 reached this extent of macrosteatosis. However, all mice on WD demonstrated microsteatosis, in contrast to the LF diet-fed mice or WD-fed mice treated with INT-767. Coincidently with this increase in steatosis was a drastic increase in hepatic foci of inflammatory cells and lipogranulomas in WD-fed mice. These features were observed to a lesser extent in WD-fed mice treated with INT-767.

2.5. INT-767 treatment arrests WD-induced liver fibrosis: WD-fed mice developed significant liver fibrosis, and treatment with INT-767 nearly abrogated the development of fibrosis, which was assessed by Picro-Sirius Red staining of collagen fibers (Figure 3C), label-free ratiometric TPE-SHG imaging (Figure 3D), and collagen-I and collagen-III protein immunohistochemistry (Figure 3E). The suppression of fibrosis following INT-767 intervention was associated with reduced expression of α-SMA and TGF-β (Figure 3F), suggesting that arrest of fibrosis development may have been mediated by effects on hepatic stellate cell function (32).

2.6. INT-767 induces pronounced alterations in serum, liver, and intestine bile acid composition: Treatment with INT-767 blocked WD-induced increases in both total serum bile acid levels, and the serum levels of individual bile acids: TCA, T-α-MCA, T-β-MCA, T-DCA, T-HDCA, T-UDCA and T-MDCA (Figure 4A). INT-767 treatment of LF mice produced variable effects on individual bile acid serum levels but did not affect
levels of total serum bile acids (Figure 4A). In contrast to the WD effects on serum bile acids, total hepatic bile acid levels were not affected by WD feeding. Nevertheless, INT-767 treatment significantly suppressed total bile acid levels in LF- and WD-fed mice and produced variable effects on individual bile acid species, including TCA, T-α-MCA, T-β-MCA, T-DCA, T-UDCA, and T-MDCA (Figure 4B). When expressed as relative composition, INT-767 treatment increased the percentage of more hydrophilic bile acids, such as T-β-MCA and T-α-MCA, whereas it markedly decreased the percentage of the less hydrophilic bile acid TCA in both LF- and WD-fed mice (Figure 4C). As a result, there was a significant decrease in the hydrophobicity index of the liver bile acid composition, making it more hydrophilic (Figure 4C). INT-767-mediated decreases in liver bile acid levels are consistent with, and most likely mediated by, a combination of decreased expression of liver bile acid synthesis enzymes (CYP7A1, CYP8B1) (Figure 1A), increased expression of liver bile acid transporter for excretion (BSEP), decreased expression of liver bile acid transporters for reabsorption (NTCP, OATP), and increased expression of bile acid transporter for efflux to the blood circulation (OSTβ) (Figure 4D).

Similar changes were observed in ileal bile acids with reduced levels in total bile acids or almost all individual bile acid species measured in the ileum of INT-767-treated mice, despite elevated expression of the apical transporter ASBT. We also found an increased percentage of hydrophilic bile acids, which contributed to the decreased hydrophobic index in the ileum from INT-767 treated groups (Figure 4E). We did not find detectable amounts of TLCA or LCA in the ileum or liver following INT-767 treatment.
As shown above, treatment with INT-767 caused a significant decrease in the hydrophobicity of liver and intestinal bile acid composition. In spite of increased *Npc1l1* mRNA expression (data not shown), decreased hydrophobicity of liver and intestinal bile acid composition will lead to decreased intestinal cholesterol absorption (33), therefore reducing systemic cholesterol and liver cholesterol levels.

2.7. Treatment with INT-767 reverses WD-induced decreases in liver AMPK-SIRT1-PGC-1α-SIRT3 pathway: Mice fed WD had marked decreases in expression of SIRT1, PGC-1α, and SIRT3 (*Figure 5A*). These alterations imply decreased mitochondrial biogenesis and mitochondrial function. We did not detect any alterations in mitochondrial number as determined by mitochondrial DNA/nuclear DNA ratio (*Figure 5B*). However, WD induced a significant decrease in mitochondrial complex I activity (*Figure 5C*). Treatment with INT-767 reversed the decreases in phospho-AMPK, SIRT1, PGC-1α, and SIRT3 (*Figure 5A*), and significantly increased complex IV activity with a trend towards improving complex I activity (p=0.07) (*Figure 5C*).

2.8. INT-767-mediated inhibition of NAFLD and NASH development are FXR-dependent: Previous studies have shown that the FXR agonist OCA inhibits the development of NAFLD and NASH (10) and that the TGR5 agonist INT-777 inhibits the development of NAFLD (19). To test whether the beneficial effects of INT-767 treatment are attributable to FXR or TGR5 agonism, *Fxr*-null mice were fed a WD and treated with either vehicle or INT-767.
The decrease in steatosis induced by INT-767 treatment was FXR-dependent, as determined by H&E staining (Figure 6A), and liver triglyceride and cholesterol composition analysis (Figure 6B). In addition, the beneficial effects of INT-767 preventing liver fibrosis were also dependent on FXR, as determined by Picro-Sirius Red staining (Figure 6C), label-free imaging with SHG microscopy (Figure 6D), type I collagen and type III collagen immunohistochemistry (Figure 6E-F), quantitative collagen measurements by LC-MS/MS (Figure 6G), and expression of pro-fibrotic TGF-β and α-SMA mRNA levels (Figure 6H).

Likewise, although treatment of WD-fed mice with INT-767 increased p-AMPK, PGC-1α, SIRT3, and MnSOD, these effects were not observed in Fxr-null mice treated with INT-767 (Figure 6I). Furthermore, treatment with the FXR agonist OCA (INT-747), but not the TGR5 agonist INT-777, increased PGC-1α and SIRT3 in the livers of WD-fed mice (Figure 6J).

2.9. INT-767-mediated inhibition of NAFLD and NASH development are TGR5-independent: To test whether the beneficial effects of INT-767 treatment are also dependent on TGR5 agonism, we fed Tgr5-null mice a WD and treated them with either vehicle or INT-767. TGR5-null mice showed no significant difference in steatosis and liver fibrosis from wild-type mice on WD. However, to our surprise, INT-767 treatment still resolved the steatosis and fibrosis in the WD-fed NASH model in Tgr5-null mice (Figure 7A-B). To clarify the contribution of TGR5 agonism, we compared INT-767 with
other two agonists, FXR specific INT-747 and TGR5 specific INT-777 in WD-induced NASH development. As expected, both INT-747 and INT-767 were beneficial in controlling liver injury and liver fibrosis, although INT-767 appeared to show better protection than INT-747 (Figure 7C-D). On the other hand, INT-777 treatment failed to decrease the liver injury and liver fibrosis, indicating that a TGR5 agonism alone is not sufficient in this NASH model (Figure 7C-D). Pathak et al. reported that FXR can induce ileal TGR5 (27). However, we did not find in our model that INT-767 can increase TGR5 expression in the ileum, or FXR deficiency can lower ileal TGR5 (Supplementary Figure 2).

2.10. INT-767 modifies gut microbiota: To understand the role of gut microbiota in this NASH model, bacterial communities inhabiting the cecum and colon were profiled in all treatment groups by 16S rRNA gene sequencing. Both the overall composition (Figure 8A) and biodiversity (Supplementary Figure 3A) of the microbiota were affected to varying degrees by diet, FXR genotype, and INT-767 treatment. Based on PERMANOVA tests of overall community composition (i.e., beta-diversity), the colonic microbiota (p<0.00001) were altered to a greater extent by these factors than were cecal microbiota (p=0.10). Furthermore, diet (LF vs. WD; p=0.0050) and FXR genotype (p<0.00001) were more significantly associated with colonic microbiota composition than was INT-767 treatment (p=0.38; Figure 8A). Nonetheless, pairwise comparisons of both WT WD vs. WT WD+INT767 (p=0.0066) and FXR KO WD vs. FXR KO WD+INT767 (p=0.0079) indicated that INT-767 treatment induced significant shifts in colon microbiota of WD-fed animals, regardless of FXR genotype. However, WT LF and
WT WD+INT767 treatment groups also differed in microbiota composition (p=0.028), indicating that INT-767 did not completely reverse WD-induced dysbiosis.

In terms of individual operational taxonomic units (OTUs), multiple phylum- and genus-level taxa were altered in the colonic microbiota of WD-fed WT animals, compared with LF-fed WT animals (**Figure 8B** and **Supplementary Figure 3B**). The most notable change was in the phylum Bacteroidetes (**Figure 8B**), which was greatly increased in WD-fed animals (27.8% vs 9.7% relative abundance in WT WD vs WT LF groups; p=0.022). These changes were due primarily to shifts in the S24-7 group of Bacteroidetes (23.0% vs. 9.6%; p=0.0043). Conversely, WD-feeding was associated with slightly diminished Firmicutes (69.2% vs 75.8%; p=0.041), including genera such as *Allobaculum* (1.2% vs. 4.2%; p=0.0022) and *Lactobacillus* (0.37% vs 12.8%; p=0.04). However, some groups of Firmicutes, such as *Roseburia* (0.71% vs 0.16%; p=0.0022) were increased in abundance in WD-fed animals. Treatment of WT WD mice with INT-767 (i.e., WT WD+INT767) reversed most of these effects (**Figure 8B** and **Supplementary Figure 3B**), resulting in Bacteroidetes and Firmicutes abundances that were more similar to WT-LF mice. In contrast, INT-767 treatment of FXT KO WD animals (i.e., FXR KO WD+INT-767) exaggerated the effects of WD feeding, as evidenced by elevated Bacteroidetes (48.3% vs 30.1% for FXR KO WD+INT-767 vs FXR KO WD; p=0.016) and diminished Firmicutes (46.8% vs. 50.7%; p=0.056). These results are consistent with the phenotypic and molecular data presented above, which indicate that FXR is required for INT-767 activity.
We next examined the relationships between intestinal bile acid pools and gut microbiota, in the WT-LF, WT-WD, and WT-WD-INT767 treatment groups, by systematically performing Spearman rank correlation tests between the abundances of bile acid species and microbial taxa. Distinct patterns of association were observed in this analysis (Figure 8C), with the colon exhibiting more significant associations than the cecum. Within the colon, several groups of the phylum Firmicutes, most notably Ruminococcaceae and Coprococcus were significantly negatively correlated with unconjugated primary and secondary bile acids. In contrast, members of the phylum Bacteroidetes, such as the Bacteroides, Parabacteroides, and S24-7 were positively correlated with these same unconjugated bile acids.

2.11. INT-767 treatment reduces MCD-induced liver steatosis but not fibrosis: To further investigate how INT-767 exerts its anti-fibrosis role, we used another diet, MCD, to generate NASH in mice (30). MCD-induced liver fibrosis does not seem to depend on liver steatosis (34). After 12-weeks on MCD diet, severe fibrosis developed in the liver as assessed by Picro-Sirius Red staining (Figure 9B). However, although INT-767 significantly reduced steatosis (Figure 9A), MCD-induced hepatic fibrosis was not affected by INT-767 treatment (Figure 9B).

2.12. FXR and TGR5 proteins are expressed in liver biopsy tissue obtained from donors with no liver disease and subjects with NAFLD and NASH: H&E and Masson’s trichrome stains showed clear evidence of steatosis and fibrosis in liver sections from subjects with NAFLD and NASH, which are lacking from donors without
liver disease (Supplementary Figure 4A). SHG microscopy, which images fibrillary collagens in a label-free manner, further documented marked differences in the degree of fibrosis between diseased and non-diseased human liver (Supplementary Figure 4A). FXR and TGR5 were detected in liver sections from both NASH and non-diseased donor patients by immunohistochemistry. FXR was predominantly expressed in hepatocyte and cholangiocyte nuclei in sections from NASH patients and showed a significant increase in staining intensities compared to non-diseased controls (p=0.004). Membranous TGR5 staining was selectively localized to cholangiocytes in the bile ducts from both non-diseased and NASH patients. The significantly increased number of TGR5 positive cholangiocytes in NASH liver sections compared to controls (p=0.0012) reflects ductular reaction (Supplementary Figure 4B).
3. Discussion

The prevalence of NASH is increasing worldwide in part due to the increased prevalence of obesity and diabetes. There are currently no pharmacological treatments for NASH. Here we report that C57BL/6J male mice fed a WD composed of milk fat, sucrose, and cholesterol, as used in atherosclerosis models, develop NASH features, including hepatic steatosis, inflammation, and fibrosis, as determined by classical histological stains and biochemical assays as well as novel and label-free microscopy imaging techniques including THG, SHG, and FLIM. In this study, we found that the FXR-TGR5 dual agonist INT-767 prevents the progression of WD-induced liver injury. This effect is dependent on FXR but not TGR5.

Although INT-767 was found to improve liver steatosis in different models, whether it can also attenuate liver fibrosis, a hallmark of NASH, has not been fully explored (21,26,27,35). In this study, we employed different methods to study the anti-fibrotic effects of INT-767 treatment in a NASH model. Conventional Picro-Sirius Red staining showed decreased fibrillar collagen deposits in the treated liver, and immunohistochemistry confirmed a reduced accumulation of collagen I and III after INT-767 treatment. Furthermore, we used mass spectrometry to quantify individual collagen subunits and found decreased expression of collagen 1α1, collagen 1α2, and collagen 3α1 chains in the treated liver. Finally, we used a multiphoton microscope equipped with DIVER detector for SHG imaging that can distinguish fibrillary collagen accumulation even in the early stages of fibrosis, unlike standard histological analysis (36-38). The
SHG imaging clearly showed decreased fibrosis in INT-767-treated mice. The anti-fibrotic role of INT-767 is remarkably similar to the beneficial effects induced by OCA treatment in animal models (39,40) and patients with NASH (10,12). Recently, in a rabbit model of high fat diet-induced NASH, INT-767 was shown to prevent liver fibrosis (24). Using ob/ob mice fed the high fat with trans fat, cholesterol, and fructose, INT-767 could also reverse NASH (25). Consistent with these findings, our study confirmed that INT-767 treatment can improve liver fibrosis in a mouse model of WD-induced NASH. Since INT-767 treatment was started after 3 months on WD, when liver injury was already established, our data indicate that INT-767 can arrest the progression of liver fibrosis in NASH thus indicating its therapeutic potential.

In terms of the mechanism by which INT-767 exerts its anti-fibrotic effects, it is conceivable that the agonism of either FXR or TGR5 could make contributions and if so, the dual agonism may carry an advantage to either singular agonism. In the diet-induced obesity model, steatosis is believed to be the first hit for fibrosis which appears later in disease progression (41). Both FXR and TGR5 are found to be involved in improving liver steatosis (19,42-45). However, to our surprise, we observed that improvement of NASH by INT-767 depends on activation of FXR rather than TGR5. Pathak et al. reported a FXR-dependent but TGR5-independent mechanism for INT-767 based on the TGR5 activation by FXR (27). In contrast, our data showed that FXR activation could not induce TGR5 expression. However, we cannot rule out the possibility that FXR can still induce GLP-1 secretion in the absence of TGR5 (27).
To further clarify the mechanism, we compared the different agonists in the NASH model. We found that the TGR5-specific agonist INT-777 failed to show the protective effects in the liver injury and liver fibrosis, whereas both INT-767 and FXR specific agonist OCA significantly reduced the liver injury and fibrosis. These results suggest that TGR5 agonism alone is not enough to treat the NASH, although it can reduce hepatic steatosis in other reports (19), and that FXR activation plays a major role in INT-767 to treat the NASH.

Other factors such as gut microbiome which is largely involved in host metabolism regulation, may act as additional possible contributor to our findings. As expected, WD-fed mice exhibited markedly altered gut microbiota, especially within the colon. WD-feeding was characterized by elevated colonic Bacteroidetes (e.g., S24-7) and reduced Firmicutes (e.g., Allobaculum spp. and Christensenellaceae spp.), compared with LF animals. However, WD-induced dysbiosis was reduced by INT-767 treatment, which prevented the changes in Bacteroidetes and Firmicutes levels observed in the untreated WD group. Although we cannot rule out a direct effect of INT-767 on the gut microbiota, INT-767 did not restore WD-induced microbial dysbiosis in Fxr-null mice, suggesting that host-dependent bile-acid signaling pathways are a determinant of gut microbiota composition. Overall, these results imply that changes in the gut microbiota in response to WD do not simply result from direct effects of elevated nutrient availability to resident microbial communities, but that diet-induced functional modification(s) of the gut-liver axis play a larger role in determining microbiome composition and function.
In our study, we also explored the FXR-dependent mechanisms lowering hepatic lipids. We found that INT-767 markedly inhibits expression of hepatic Cyp7a1 and Cyp8b1, two major enzymes involved in bile acid synthesis. This led to decreased total bile acid content and caused marked modifications in liver and intestinal bile acid composition with decreased hydrophobicity index, known to inhibit intestinal cholesterol absorption (33). INT-767-induced FXR activation prevented SREBP-1c, ChREBPα and ChREBPβ mediated fatty acid synthesis, CD36 and FABP1 mediated fatty acid uptake, and LOX-1 mediated cholesterol uptake in the liver. LXRα may be also involved, as lowering cholesterol can decrease LXRα activity, which in turn reduce its target SREBP-1c expression. INT-767 activates FXR in both liver and intestine, in contrast with the findings that intestinal FXR inhibition is beneficial in preventing NASH (46). While we expect data from tissue-specific FXR knockout mice to provide a more specific direction, we speculate that intestinal FXR is dispensable for INT-767 function, as one of bile acid synthesis enzymes, Cyp8b1, does not require intestinal FXR (33).

In addition, INT-767 increased p-AMPK, SIRT1, PGC-1α, and SIRT3, which are master regulators of mitochondrial biogenesis and mitochondrial function. Despite no changes in mitochondrial DNA/nuclear DNA ratio, INT-767 improved mitochondrial function, as evidenced by the near reversal of WD-induced decrease in complex I activity and a significant increase in complex IV activity. Recent studies indicate that improving
mitochondrial function per se may mediate anti-inflammatory, anti-oxidative, and anti-fibrotic effects (47-50).

The anti-fibrotic effects of INT-767 may depend on the trigger of fibrosis in each model. In the MCD-induced NASH model, we observed a different effect with INT-767. It is known that MCD-induced liver fibrosis is independent of steatosis (34). In our study, although steatosis was almost completely prevented by INT-767, MCD-induced liver fibrosis still progressed in the presence of INT-767, unlike WD-induced NASH, suggesting that its anti-fibrotic effects depend on specific mechanisms of fibrosis development in each model.

In summary, the present results indicate that WD in C57BL/6J mice induces steatosis, inflammation, and fibrosis (NASH), associated with mitochondrial dysfunction and gut microbiota dysbiosis. Prevention or treatment with the dual FXR-TGR5 agonist INT-767 can arrest the progression of WD-induced NASH in mice, mediated largely by FXR-dependent, TGR5-independent mechanisms.
4. Experimental Procedures

4.1. Liver biopsy samples from human subjects: We obtained deidentified liver biopsy samples (FFPE) from the pathological archives at the University of Colorado. These samples were from healthy donors as well as subjects with stage 0-4 liver fibrosis, as scored blindly by the liver pathologist (D.E.K.) based on the Brunt and Kleiner scoring systems (51,52). The samples were stained with H&E, Masson’s trichrome, and imaged by two-photon excitation (TPE)-second harmonic generation (SHG) microscopy for label-free imaging of fibrosis. We also performed immunohistochemistry for FXR and TGR5 expression in the liver samples.

4.2. TGR5 and FXR Immunohistochemistry: Briefly, staining was performed on 5 um formalin-fixed paraffin-embedded sections with anti-TGR5 rabbit polyclonal antibody (1:200 for 2 h; Sigma-Aldrich, product number HPA062890) or anti-FXR monoclonal antibody (R&D, product number PP-A9033A-00). Image analysis was performed using ImageScope (Leica Biosystems Pathology Imaging) with positive pixel count algorithms for TGR5 staining and nuclear algorithm for FXR staining.

4.3. Animal Models: The mouse studies were performed with wild-type C57BL6/J, FXR knockout (Fxr-null) male mice on the C57BL6/J background obtained from Jackson Laboratories, and TGR5-null mice backcrossed to the C57BL6/J background (a gift from Professor Johan Auwerx, École Polytechnique Fédérale de Lausanne, Switzerland). The mice were housed 4 mice per cage, kept at room temperature using a 12-hour dark to 12-hour light cycle, and allowed free access to food and water.
For the intervention study, eight-week-old male C57BL/6J mice were fed a WD consisting of 42 kcal % milkfat, 42.7 kcal % carbohydrate, 15.2 kcal % protein and supplemented with 0.2% cholesterol (Harlan Teklad TD.88137) or a low-fat control diet (LF) consisting of 13 kcal % fat, 67.9 kcal % carbohydrates, 19.1 kcal % protein, without added cholesterol (Harlan Teklad TD.08485) for 3 months to induce liver injury, at which time the mice were maintained on the same diets without supplementation or were fed the same diets supplemented with 30 mg/kg body weight INT-767 (20) for an additional 3 months. The mice were evaluated at 8 months of age, at the end of the 6-month treatment period.

To determine the role of FXR signaling in response to INT-767 treatment, wild-type eight-week-old male C57BL/6J mice and age/gender-matched Fxr-null mice (53) with the C57BL/6J background were fed WD, with or without supplementation with 30 mg/kg body weight INT-767, for 5 months. These mice were evaluated at 7 months of age, at the end of the 5-month treatment period.

To determine the role of TGR5 signaling in response to INT-767 treatment, we used eight-week-old male TGR5-null mice fed WD with or without supplementation with 30 mg/kg body weight INT-767. These mice were evaluated at 8 months of age, at the end of the 6-month treatment period.
To compare the effects of different agonists, eight-week-old male C57BL/6J mice were fed WD alone or WD supplemented with INT-767, or INT-747 (Intercept, New York, NY) or INT-777 (Intercept, New York, NY), at 30 mg/kg body weight for 6 months.

To determine if INT-767 was also effective in a different NASH model, eight-week-old male C57BL/6J mice were fed a methionine and choline deficient diet (MCD, Research Diets A02082002B) with or without 30 mg/kg body weight INT-767 for 12 weeks.

At the end of the treatment periods, the mice were anesthetized, blood (serum) was obtained from the vena cava, then the liver and intestine were harvested and processed for the biochemical and imaging studies detailed below.

4.4. Blood chemistry: Blood glucose levels were measured with a Glucometer Elite XL (Bayer, Tarrytown, NY). Plasma triglyceride and cholesterol were measured with kits from Wako Chemical (Richmond, VA). Plasma liver enzymes were analyzed with kits from Bioassay Systems (Hayward, CA).

4.5. RNA extraction and real-time quantitative PCR: Total RNA was isolated from the livers using Qiagen RNeasy mini kit (Valencia, CA), and cDNA was synthesized using reverse transcript reagents from Bio-Rad Laboratories (Hercules, CA). Quantitative real-time PCR was performed as previously described (54-57), and expression levels of target genes were normalized to 18S level. Primer sequences are listed in Supplementary Table 2.
4.6. Western blotting: Western blotting was performed as previously described (54-57). An equal amount of total protein was separated by SDS-PAGE gels and transferred onto PVDF membranes. After HRP-conjugated secondary antibodies, the immune complexes were detected by chemiluminescence captured on UVP Biospectrum 500 Imaging System (Upland, CA), and the densitometry was performed with ImageJ software. Antibodies against AMPK (07-350, Millipore, Billerica, MA), Sirtuin 1 (05-1243, Millipore), PGC-1α (AB3242, Millipore), p-AMPK (Thr172, 2531, Cell Signaling, Danvers, MA), Sirtuin 3 (5490, Cell Signaling), and SOD2 (ADI-SOD-110, Enzo Life Sciences, Farmingdale, NY) were used for Western blotting.

4.7. Autofluorescence FLIM, SHG, and THG measurements using DIVER microscope: To determine the effects of WD and treatment with INT-767 on lipids and fibrillar collagens, autofluorescence FLIM, second harmonic generation (SHG), and third harmonic generation (THG) signals were acquired using the DIVER (Deep Imaging via Enhanced-Photon Recovery) microscope developed at the Laboratory for Fluorescence Dynamics (LFD), University of California at Irvine, CA, as detailed in Supplementary Methods.

4.8. Histological stains and assessment: 5 μm paraffin sections were used for histological stains. Hematoxylin and eosin (H&E) stained sections were scored for liver injury according to the liver scoring system similar to that of Kleiner et al (52) but modified for mouse liver (31). For quantification of the fibrosis following PSR staining,
ten polarized light images were made in a “tiling” fashion across each PSR stained slide and then quantified using the 3I Slidebook program (3I, Denver, Colorado) to arrive at the PSR positive pixels per 100x field for that slide. Immunofluorescent staining was performed for: Perilipin 2 (20R-AP002, Fitzgerald Industries), CD3epsilon, Collagen type 1, and Collagen type 3, all as previously described (58) with detection using secondary antibodies conjugated with Alexafluor 488 or Alexafluor 594 at dilutions of 1:500 and 1:250, respectively and nuclear staining with DAPI (4′, 6-diamidino-2-phenylindole, Sigma Chemical Co.). Immunofluorescence images were captured on a Nikon Diaphot fluorescence microscope and digitally deconvolved using the No Neighbors algorithm (Slidebook; Denver, CO) as described previously (59).

4.9. Mouse collagen mass spectroscopy: Tryptic digests of mouse liver samples were analyzed by LC-MS/MS using multiple reaction monitoring (MRM) targeted mass spectrometry on a triple quadrupole mass spectrometer, as detailed in Supplementary Methods and Supplementary Table 3.

4.10. Hepatic triglyceride and cholesterol concentration and lipidomics: Total hepatic triglyceride and cholesterol levels were quantified as described previously (60), and as detailed in Supplementary Methods.

4.11. Serum, liver, and intestine bile acid composition analysis: Concentrations of bile acids were determined by an Acquity UPLC/Xevo G2 QTOFMS system (Waters Corp.) with an ESI source. An Acquity BEH C18 column (100x2.1mm internal diameter,
1.7 mm, Waters Corp., Milford, MA) was applied for chromatographic separation. A mixture of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was used as the mobile phase. The gradient elution was started from 80% (A) for 4 min, decreased linearly to 60% (A) over 11 min, to 40% (A) over the next 5 min, to 10% (A) for the succeeding 1 min, and finally increased to 80% (A) for 4 min to re-equilibrate the column. The column temperature was maintained at 45°C, and the flow rate was 0.4 ml/min. Mass spectrometry detection was operated in negative mode. A mass range of m/z 50–850 was acquired (61, 62).

4.12. Microbiome analysis: Broad-range PCR and sequencing of 16S rRNA genes followed our previously described protocols (63, 64). In brief, DNA was extracted from 50–100 mg of cecal or colon contents using the QIAmp Stool DNA isolation kit (QIAGEN, Inc., Carlsbad, CA). 16S rRNA gene PCR amplicons were generated using barcoded primers (65) targeting the V3V4 variable region: primers 338F (5’ ACTCCTACGGGAGGCAGCAG) and 806R (5’ GGACTACHVGGGTWTCTAAT) (66, 67). Sequencing was performed on the Illumina MiSeq platform using a 600-cycle version 3 reagent kit, version v2.3.0.8 of the MiSeq Control Software, and version v2.3.32 of MiSeq Reporter. Demultiplexed paired-end reads were assembled using phrap (68) and pairs that did not assemble were discarded. Potential chimeras were identified and discarded using UCHIME (usearch6.0.203_i86linux32) (69) and the Schloss (70) Silva reference sequences. Assembled sequences were aligned and classified with SINA (1.3.0-r23838) (71) using the Silva 115NR99 database (72) as the
reference. Operational taxonomic units (OTUs) were produced by clustering sequences with identical taxonomic assignments.

4.13. **Statistical analysis:** Results are presented as the means ± SEM for at least three independent experiments. Data were analyzed by ANOVA and Student–Newman–Keuls tests for multiple comparisons or by t-test for unpaired data between two groups (GraphPad Prism). Statistical significance was accepted at the p<0.05 level. Microbiome analyses used Explicet (73) and the R statistical software package (74). The relative abundance of each taxon was calculated as the number of 16S rRNA sequences of a given taxon divided by the total number of 16S rRNA sequences in a patient’s sample. Differences in overall microbiome composition (i.e., beta-diversity) between subsets were assessed by non-parametric multivariate analysis of variance (PERMANOVA) test using Morisita-Horn dissimilarities; p-values were estimated through 1,000,000 permutations. Shannon diversity, Shannon evenness, and richness (Sobs) were calculated using rarefaction and compared across groups through analysis of variance (ANOVA) tests. Comparisons of relative abundance across groups were conducted by non-parametric Kruskal-Wallis tests. Associations between cecal bile acid species concentrations and relative abundances of genus-level bacterial taxa were assessed by Spearman rank-sum correlation tests.

4.14. **Study approval:** Animal studies and relative protocols were approved by the Animal Care and Use Committee at the University of Colorado AMC, Aurora, Colorado, and Georgetown University, Washington, DC. All animal experimentation was
conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD).
Data Availability

All the data described in the article are located within the article and/or its supporting information.
Acknowledgments

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Conflict of Interest

Dr. Luciano Adorini is an employee of Intercept. These studies were in part supported by a medical school Investigator Initiated Study (IIS) grant to Moshe Levi. The authors otherwise have no other conflict of interest.
Figure Legends

**Figure 1**: INT-767 regulates FXR target genes in the liver and regulates both FXR and TGR5 target genes in the ileum. **A)** INT-767 decreases liver *Cyp7a1* and *Cyp8b1* mRNA expression and increases liver *Shp* and *Bsep* mRNA expression. **B)** INT-767 increases the mRNA expression of FXR targets in the ileum: *Fgf15*, and *Shp*, and **C)** TGR5 target genes in the ileum, *Gcg* mRNA. N= 6 mice. * = p< 0.05 WD vs. LF; ** = p< 0.05 WD + INT-767 vs. WD.

**Figure 2**: INT-767 prevents WD-induced steatosis, triglyceride, and cholesterol accumulation in the liver. **A)** Steatosis as shown by H&E staining and liver injury scoring in mice on WD for 3-months compared to those on control low fat diet. **B-D)** Steatosis as determined by (B) H&E stain, (C) perilipin-2 (PLIN2) immunohistochemistry for lipid droplets, (D) label-free imaging with Fluorescence Lifetime Imaging Microscopy (FLIM) and Third Harmonic Generation (THG) microscopy. The red areas in FLIM images show the lipid droplets identified by the long lifetime in FLIM images and their boundaries can be seen in THG images. The histogram shows the changes in the droplet sizes calculated from FLIM data. The p-value on the histogram: p <0.0001 LF vs WD and WD vs WD+INT-767. **E)** Triglyceride content as determined by GC-MS. **F-G)** Mediators of fatty acid synthesis; SREBP-1c, SCD1, ChREBP-α, and ChREBP-β with mRNA levels as determined by RT-qPCR. **G)** Mediators of fatty acid uptake; *Cd36* and *Fabp1* with mRNA levels as determined by RT-qPCR. **H)** Liver cholesterol ester content as determined by GC-MS. **I-J)** A mediator of cholesterol uptake, LOX-1, mediators of
cholesterol synthesis including SREBP-2 and HMG-CoA reductase, and mediator of cholesterol efflux including ABCG5 and ABCG8 mRNA levels are shown, as determined by RT-qPCR. N= 6 mice. * = p< 0.05 WD vs. LF; ** = p< 0.05 WD + INT-767 vs. WD.

Size bars=100 microns.

**Figure 3**: INT-767 prevents WD-induced inflammation and fibrosis in the liver. **A-B)** Inflammation as determined by CD3 immunohistochemistry, and MCP-1, TNF-α, and IL-1β mRNA levels; **C-E)** Fibrosis as determined by (C) Picro-Sirius Red staining, (D) label-free imaging with ratiometric TPE-SHG microscopy, and (E) type I collagen and type III collagen immunohistochemistry. In (D), the ratio of fraction covered by SHG (green) to the area covered by autofluorescence (red) indicated by $f_{\text{green}}/f_{\text{red}}$ shows the extent of fibrilar collagen accumulation and how INT-767 reverses the effect of WD. **F)** INT-767 decreased TGF-β and α-SMA mRNA levels. N= 6 mice. * = p< 0.05 WD vs. LF; ** = p< 0.05 WD + INT-767 vs. WD. Size bars=100 microns.

**Figure 4**: INT-767 regulates bile acid composition of serum, liver, and ileum. **A)** WD induced significant increases in serum total bile acids TCA, T-α-MCA, T-β-MCA, TDCA, T-HDCA, T-UDCA, and T-MDCA levels. Treatment with INT-767 prevented these increases and also resulted in significant decreases in serum TCA, T-α-MCA, T-β-MCA, TDCA, T-CDCA, T-HCDA, and T-MCDA levels. **B)** Treatment of WD-fed mice with INT-767 also resulted in significant alterations in liver total bile acids and individual bile acid species including TCA, T-α-MCA, T-β-MCA, T-DCA, T-UDCA, and T-MDCA. **C)** When
expressed as relative composition as shown in the pie chart, treatment with INT-767 increased the relative level of T-β-MCA and T-α-MCA but decreased that of TCA content. These changes resulted in a major decrease in the hydrophobicity index of the liver bile acid composition. D) Treatment with INT-767 increased expression of liver bile salt export pump (BSEP), decreased expression of liver bile acid reabsorption transporters (NTCP, OATP), and increased expression of bile acid efflux transporters to the blood circulation (OSTβ). E) Treatment of WD mice with INT-767 resulted in significant alterations in ileal total bile acids and in ileal individual bile acid species. The pie chart for ileum bile acid composition showed the decreased TCA and increased T-β-MCA and T-α-MCA in INT-767 treated mice, which contributed to the decreased hydrophobicity index. N= 6 mice. * = p< 0.05 WD vs. LF; ** = p< 0.05 WD + INT-767 vs. WD.

**Figure 5:** INT-767 increased mitochondrial function. A) p-AMPK, SIRT1, PGC-1α, and SIRT3 protein levels were determined by western blotting and normalization to β-actin. B) Mitochondrial DNA/nuclear DNA ratio. C) Complex I and complex IV activity were determined by kits from MitoSciences/Abcam. N= 6 mice. * = p< 0.05 WD vs. LF; ** = p< 0.05 WD + INT-767 vs. WD.

**Figure 6:** The effects of INT-767 on NASH are FXR-dependent. A-B) Steatosis as determined by H&E staining, liver triglyceride, and cholesterol content; C-H) Fibrosis as determined by (C) Picro-Sirius Red staining, (D) label-free imaging with SHG, (E)
collagen I and (F) collagen III immunohistochemistry, (G) quantitative measurement of collagen 1α1, collagen 1α2, and collagen 3α1 by LC-MS/MS, and (H) TGF-β and α-SMA mRNA levels. Size bars=100 microns. I) The effects of INT-767 to stimulate p-AMPK, PGC-1α, SIRT3, and SOD2 protein are FXR-dependent. INT-767 stimulates p-AMPK, PGC-1α, SIRT3, and SOD2 protein in wild-type mice fed a WD but not in Fxr-null mice fed a WD. J) Treatment of WD-fed mice with the FXR agonist OCA (INT-747) but not the TGR5 agonist INT-777 induces upregulation of PGC-1α and SIRT3 mRNA. N= 6 mice. * = p< 0.05 WD vs. LF; ** = p< 0.05 WD + INT-767 vs. WD. Size bars=100 microns.

Figure 7: The effects of INT-767 on NASH are TGR5-independent. A-B) Steatosis as determined by H&E staining (A) and fibrosis as determined by Picro-Sirius Red staining with subsequent quantification (B). Only groups of wild type WD (WTWD), wild type WD+ INT-767 (WTWD+INT), TGR5KO WD, and TGR5KO WD+INT-767 in the study were compared. C-D) Steatosis as determined by H&E staining with quantification of liver total injury (C) and fibrosis as determined by Picro-Sirius Red staining with quantification (D). Only groups of wild type WD (reuse of WTWD images of H&E (panel A) and polarized Picro-Sirius Red staining (panel B)) , wild type WD+ INT-747, wild type WD+ INT-767 (reuse of WTWD+INT images of H&E (panel A) and polarized Picro-Sirius Red staining (panel B)) and wild type WD+ INT-777 in the study were compared. N= 6 mice. * = p< 0.05 vs. WTWD; ** = p< 0.05 vs. TGR5KOWD. Size bars=100 microns.
Figure 8: WD and INT-767 modulate the microbiota. A) Distribution of bacterial genera in cecal and colon contents. Results of permutation-based multiple analysis of variance (PERMANOVA) tests for pairwise comparisons between treatment groups are shown by symbols above bar charts. PERMANOVA tests across all treatment groups are summarized below bar charts: “Overall” shows the p-value for tests across all five treatment groups, while “Diet”, “INT767”, and “FXR geno” summarize the p-values for multiple-factor PERMANOVA tests. •: p<0.1; *: p<0.05; **: p<0.01. B) Summary of relative abundances of the key intestinal phyla, Bacteroidetes and Firmicutes, across treatment groups. •: p<0.1; *: p<0.05; **: p<0.01. C) Heatmaps displaying Spearman rho correlation coefficients for pairwise comparisons of cecal bile acid species vs. cecal or colonic microbial taxa (top 15 most abundant genus-level taxa). Genus-level taxon names are preceded by abbreviated phylum names: Acti: Actinobacteria, Bact: Bacteroidetes, Firm: Firmicutes. *: p<0.05; #: p<0.01.

Figure 9: The effects of INT-767 on MCD induced NASH. A) Steatosis as determined by H&E staining; B) Fibrosis as determined by Picro-Sirius Red staining. Size bars=100 microns.
References


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Table 1: Metabolic parameters

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<th>LF+INT-767</th>
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<th>WD+INT-767</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>36.1±1.06</td>
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<td>Food intake (kcal/mouse/day)</td>
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<td>Liver weight/Body weight (%)</td>
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<td>Blood glucose (mg/dL)</td>
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<td>AST (U/L)</td>
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<td>Fasting serum cholesterol (mg/dL)</td>
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Data are means ± SE (n=6 mice in each group):  a p < 0.05 vs. LF,  b p < 0.05 vs. WD.
Figure 5

A

P-AMPK/AMPK protein

B

Mito DNA/Nuclear DNA

C

Complex I Activity

Complex IV Activity
Author Contributions

ML and XXW conceived and designed research; XXW, CX, AEL, SR, JL, KM, KB, DJO, ST, AD, DEK, SMH, KWK, AR, CER, and DI performed experiments; XXW, DJO, and DNF analyzed data and interpreted results of experiments; XXW write the manuscript; MC, BAJ, LA, JBK, JLM, FJG, EG and ML edited the manuscript; ML and XXW approved final version of manuscript.