



Activation of farnesoid X receptor (FXR) protects against fructose-induced liver steatosis via inflammatory inhibition and ADRP reduction



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ABSTRACT

Fructose is a key dietary factor in the development of nonalcoholic fatty liver disease (NAFLD). Here we investigated whether WAY-362450 (WAY), a potent synthetic and orally active FXR agonist, protects against fructose-induced steatosis and the underlying mechanisms. C57BL/6J mice, fed 30% fructose for 8 weeks, were treated with or without WAY, 30 mg/kg, for 20 days. The elevation of serum and hepatic triglyceride in mice fed 30% fructose was reversed by WAY treatment. Histologically, WAY significantly reduced triglyceride accumulation in liver, attenuated microphage infiltration and protected the junction integrity in intestine. Moreover, WAY remarkably decreased portal endotoxin level, and lowered serum TNF α concentration. In lipopolysaccharide (LPS)-induced NAFLD model, WAY attenuated serum TNF α level. Moreover, WAY suppressed LPS-induced expression of hepatic lipid droplet protein adipose differentiation-related protein (ADRP), down-regulation of it in mice fed 30% fructose. Furthermore, WAY repressed lipid accumulation and ADRP expression in a dose-dependent manner in palmitic acid (PA)-treated HepG2 and Huh7 cells. WAY suppressed TNF α -induced ADRP up-regulation via competing with AP-1 for ADRP promoter binding region. Together, our findings suggest that WAY, an FXR agonist, attenuates liver steatosis through multiple mechanisms critically involved in the development of hepatosteatosis, and represents a candidate for NAFLD treatment.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by the accumulation of triglycerides in hepatocytes exceeding 5% of the liver weight in the absence or with little consumption of

alcohol [1]. NAFLD represents a range of pathologic features, from simple steatosis to nonalcoholic steatohepatitis (NASH), and may progress to cirrhosis and hepatocellular carcinoma (HCC) [1]. NAFLD currently affects 20%–30% of adults and 10% of children in industrialized countries [2]. The mechanisms involved in NAFLD are not fully understood.

Farnesoid X receptor (FXR; NR1H4), a member of the nuclear receptor superfamily, is mainly expressed in the liver, intestine, kidneys and, to a lower extent, adipose tissue [3]. It regulates expression of a variety of genes critically involved in the control of bile acids, lipid, and glucose homeostasis [4]. Accumulated evidence suggests that the FXR dependent pathway protects the liver from fatty accumulation, and this protective effect was abolished in FXR null mice [5,6]. Zhang et al. showed that a potent synthetic FXR agonist, WAY-362450 (WAY), protected against NASH in a

Abbreviations: NAFLD, nonalcoholic fatty liver disease; FXR, farnesoid X receptor; LPS, lipopolysaccharide; WAY, WAY362450; ADRP, adipose differentiation-related protein; PA, palmitic acid; AP-1, activator protein-1; qPCR, quantitative polymerase chain reaction; NASH, nonalcoholic steatohepatitis; MyD88, myeloid differentiation factor 88; IRF3/7, interferon regulatory factor 3/7; MCP-1, monocyte chemoattractant protein-1; TG, triglyceride; ChIP-seq, chromatin immunoprecipitation-sequencing.

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model caused by methionine and choline-deficient (MCD) diet [7]. Our group showed that WAY attenuated alcohol-induced liver injury, steatosis and cholestasis [8].

Growing evidence suggests that the epidemic of NAFLD is closely intertwined with the Westernization of dietary patterns, especially an increasing intake of fructose [9]. Excess fructose consumption has been considered to be a critical factor in the development of NAFLD directly (through hepatotoxic damage) and indirectly (through metabolic adverse effects) [10]. In addition, the relationship between fructose and the gut-liver axis has attracted more attentions recently. And it is proposed that high fructose intake increases gut permeability, and promotes intestinal bacterial dysbiosis [11,12]. The combined effects result in increased translocation of bacterial endotoxin and subsequently the elevation of endotoxin in the portal vein. The endotoxemia may in turn lead to chronic inflammation, immune dysregulation, and finally metabolic abnormalities in the liver, seen in NAFLD [12]. However, no studies have investigated the role of FXR in fructose-induced NAFLD model.

The adipose differentiation-related protein (ADRP) was first characterized during a search for genes expressed in an early phase of adipocyte differentiation [13]. ADRP is a widely distributed lipid droplet protein, and plays an essential role in lipid metabolism [14]. Overexpression of ADRP stimulates fatty acid uptake and triglyceride formation, whereas inhibition of ADRP expression decreases lipid accumulation [15]. ADRP was found abundantly presented on the surface of lipid droplets in hepatocytes from fatty liver patient [16]. However, it is unclear whether FXR has any effect on ADRP expression or function in hepatic steatosis.

In this study we used an FXR agonist, WAY, as a tool to investigate the significance of FXR in mediating liver steatosis. Our findings revealed that FXR activation corrected hypertriglyceridemia in fructose feeding or LPS-treated mice and in PA-treated hepatoma cells. Furthermore, we found that the protective effect of FXR activation is mediated through suppressing ADRP expression by blocking the binding of AP-1 to ADRP promoter, and through other mechanisms, such as reducing release of inflammatory

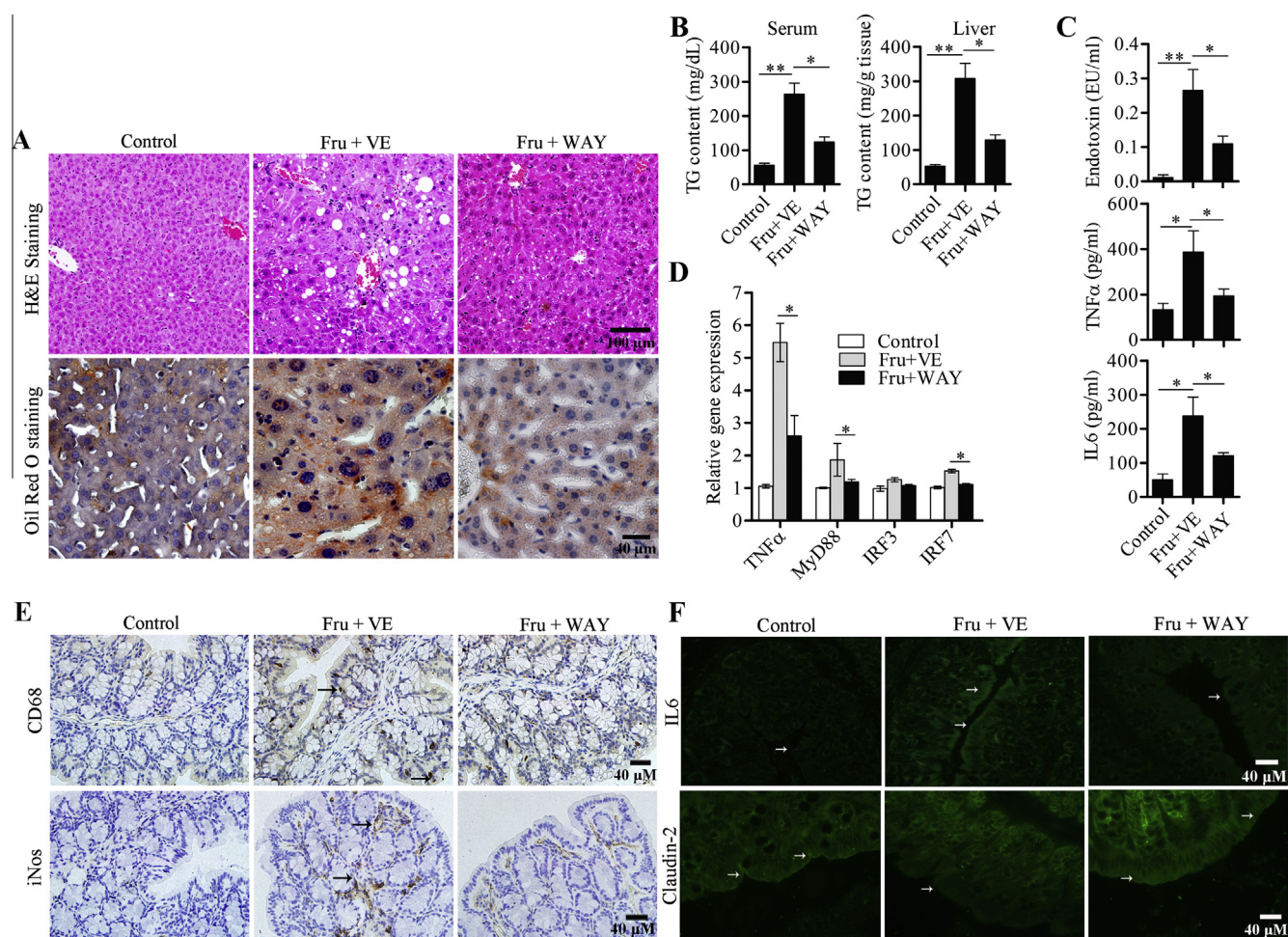


Fig. 1. WAY-362450 attenuated fructose-induced liver steatosis through reducing portal vein endotoxin level, decreasing inflammation, and protecting intestine barrier integrity. (A) Representative photographs of hematoxylin and eosin staining and Oil Red O staining of livers from mice are shown. 3 sections/samples and $n = 8$ mice/group. Scale bar: 100 μ m for HE staining; 40 μ m for Oil Red O staining. (B) Quantitative analysis of serum or hepatic triglyceride content (mg triglyceride/dl serum or mg triglyceride/g liver protein). (C) Upper: Endotoxin concentration (EU/ml) in plasma obtained from portal vein of mice. $n = 20$ /group; Middle: Quantification of TNF α levels in serum by enzyme-linked immunosorbent assay; Bottom: Quantification of IL6 levels in serum by enzyme-linked immunosorbent assay. Note that WAY treatment abolished the up-regulation of IL6 in serum induced by 30% fructose feeding. (D) Quantification of relative expression level of genes involved in LPS/TLR4 pathway. qRT-PCR was performed to measure the relative gene expression of TNF α , MyD88, IRF-3 and IRF-7. Note that fructose up-regulated the transcription level of TNF α and TLR4 adapter proteins, MyD88 and IRF-7, but not IRF-3, whereas, WAY treatment abolished the TNF α , MyD88 and IRF-7 over-expression. (E) Immunostaining of duodenum tissue with antibodies of Cd68 and iNos (marker for macrophage activation, black arrow). (F) Immunostaining of duodenum tissue with antibodies of IL6 (white arrow), and Claudin-2 (marker for tight junction, white arrow). Scale bar: 40 μ m. Fru, Fructose; VE, vehicles; WAY, WAY-362450. Error bars represent SEM. * $p < 0.05$; ** $p < 0.01$.

cytokines, decreasing portal endotoxin level, and protecting intestine membrane integrity.

2. Materials and methods

2.1. Animals and treatments

Animal experiments were fully complied with the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23 revised 1985). Mice were fed tap water or water containing 30% fructose for 8 weeks [12], and then were treated with vehicle (VE, corn oil) or WAY (30 mg/kg dissolved in corn oil) administration by oral gavage, once a day for 20 days. For LPS treatment, 6 h prior to LPS treatment, mice were pretreated with or without WAY, and then a single dose of LPS (3 mg/kg) (Sigma, St Louis, MO, USA) was injected intra-peritoneally. Blood was collected prior to sacrifice and liver tissues were harvested at indicated time points after LPS administration.

2.2. Cell culture and treatment

HepG2 and Huh7 cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (Life technologies Inc.), and cells were changed to serum-free medium 2 h prior to WAY treatment, and then WAY was added into medium to reach a final concentration of 1 μ M or 3 μ M. PA (0.4 mM) was added 4 h after adding WAY, and cells were collected after exposing to PA for 24 h.

2.3. p-c-Jun/AP-1 activity assay and chromatin immunoprecipitation (ChIP) assay

See the.

2.4. Statistical analysis

Data were expressed as means $\pm$ SEM. Statistical analysis was performed either by Student's *t* test for unpaired data or

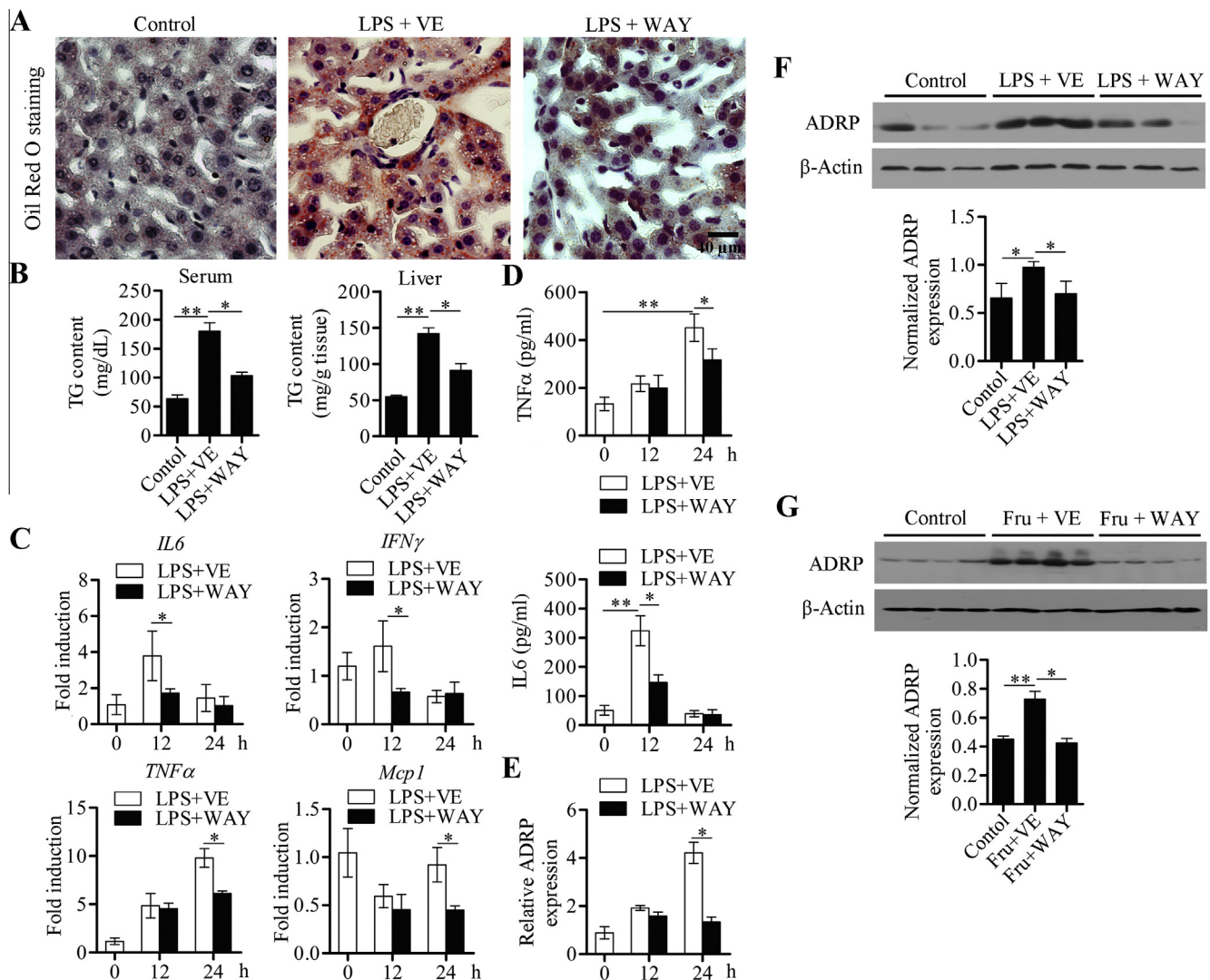


Fig. 2. WAY-362450 attenuated LPS-induced liver steatosis through suppressing ADRP expression. (A) Representative photographs of Oil Red O staining of livers from mice are shown. Note that WAY attenuated LPS induced steatosis indicated by lipid staining. 3 sections/sample and $n = 8$ mice/group. Scale bar: 40 μ m. (B) Quantification of the serum and hepatic triglyceride levels. Note that LPS induced the elevation of serum and hepatic TG content by around 3-fold. While WAY reduced the induction to less than 1-fold compared with control. (C) Quantification of pro-inflammation cytokines gene expression. Note that WAY abolished the LPS induced gene transcription of IL6 and IFN γ at 12 h, and the gene transcription of TNF α and Mcp1 at 24 h. (D) Quantification of serum TNF α (upper) and IL6 (bottom) level by ELISA. (E) Relative expression level of ADRP gene transcription. (F and G) WAY suppressed ADRP protein expression of liver tissue in both LPS-suppressed and fructose-induced steatosis mouse model. Left: Western blot image; Right: Western blot quantification. Error bars represent SEM. * $p < 0.05$; ** $p < 0.01$.

one-way ANOVA for three groups or more. $*p < 0.05$ and $**p < 0.01$ was considered as significant.

Additional materials and methods are shown in supplemental information.

3. Results

3.1. WAY-362450 attenuated fructose-induced liver steatosis through reducing portal vein endotoxin level, decreasing inflammation, and protecting intestine barrier integrity

To determine whether a FXR specific agonist, WAY-362450 (WAY), protects against fructose-induced NAFLD, a liver steatosis model was established by feeding mice 30% fructose for 8 weeks. As shown in Fig. 1A and B, no significant pathological changes were found and liver lipid staining is minimal in control liver specimens. Feeding 30% fructose for 8 weeks led to a nearly 6-fold increase of lipids contents in serum and liver respectively, and induced significant hepatic steatosis, characterized by the presence of macrovesicular and microvesicular lipid droplets in hepatocytes. Whereas, triglyceride levels were reduced to ~1-fold higher than control in serum and liver respectively in WAY treated mice, and the pathological changes were reversed accordingly.

Endotoxin and TNF α levels were elevated by ~20-fold and ~3-fold, respectively in fructose fed mice (Fig. 1C). And WAY treatment remarkably reduced serum endotoxin level, and reversed TNF α to a level comparable to the control group at both RNA and protein level (Fig. 1C and D). Furthermore, endotoxin-dependent activation of TLR4 signaling cascades was investigated in these animals. As shown in Fig. 1D, MyD88 and IRF-7 were up-regulated at the transcription level upon fructose stimulation, but not IRF-3; whereas, WAY treatment abolished the MyD88 and IRF-7 over-expression.

More importantly, we found that WAY decreased intestine permeability and macrophage infiltration. In the duodenum from fructose-fed mice, more macrophages were activated in intestinal mucosa, as indicated with CD68 and iNos staining (Fig. 1E). Meanwhile, pro-inflammatory cytokine IL6 expression level was increased; and expression level of tight junction marker, Claudin-2, was markedly reduced in intestinal epithelia (Fig. 1F). However, upon WAY treatment, the induction of inflammatory infiltration was abolished, and the reduced expression of Claudin-2 was restored.

Taken together, our results suggest that FXR agonist protects against fructose-induced liver steatosis by reducing portal vein endotoxin level, decreasing inflammation, and protecting intestine barrier integrity.

3.2. WAY-362450 attenuated lipopolysaccharide (LPS)-induced liver steatosis through reducing inflammation and suppressing ADRP expression

To further understand FXR effects on fructose-induced liver steatosis, LPS (so called endotoxin)-induced hepatic steatosis was established in mice. As shown in Fig. 2A and B, WAY significantly improved LPS-induced steatosis, indicated by less lipid accumulation in the live and reduced serum and hepatic TG contents. And WAY prevented LPS-induced liver steatosis by attenuating the expression of hepatic inflammatory cytokines. As shown in Fig. 2C, mRNA levels of pro-inflammatory cytokines, TNF α , IL6, IFN γ , and MCP1, were significantly increased at 12 h or 24 h after LPS injection, and the elevation of TNF α and IL6 expression was further confirmed by ELISA (Fig. 2D). WAY treatment reversed the changes of pro-inflammatory cytokines in LPS-treated mice.

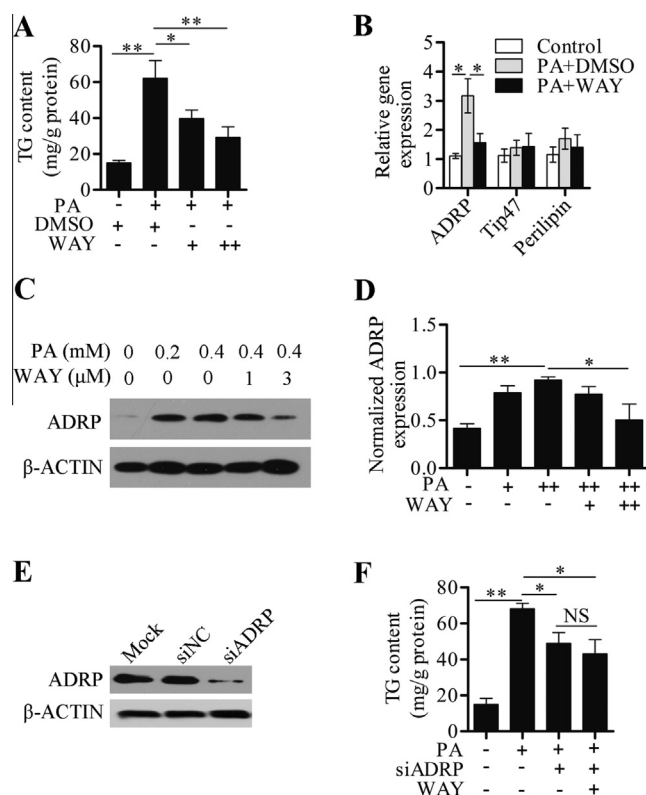


Fig. 3. WAY-362450 down-regulates ADRP expression that was induced in PA stimulated hepatocytes. (A) Quantification analysis of intracellular triglycerides content by ELISA. (B) Quantification of relative mRNA level of lipid droplets proteins, ADRP, TIP47 and Perilipin by qRT-PCR. (C and D) WAY abolished the induction of ADRP protein level induced by PA in a dose-dependent manner in HepG2 cell. (E) Specific knockdown of ADRP was confirmed by Western blot in Huh7 cells. (F) Quantitative analysis of intracellular TG content in Huh7 cells. Error bars represent SEM. $*p < 0.05$, $**p < 0.01$, NS: no significant difference.

We then examined whether WAY regulates ADRP expression in LPS-induced steatosis. As shown in Fig. 2E, mRNA level of ADRP was increased 24 h after LPS injection. And WAY treatment reversed LPS-induced ADRP over-expression. Consistent with the observation at mRNA level, WAY treatment remarkably reduced ADRP protein elevation caused by LPS injection (Fig. 2F) or fructose feeding (Fig. 2G).

3.3. WAY-362450 attenuated palmitic acid (PA)-induced lipid accumulation in hepatoma cells through specifically suppressing ADRP expression

To further explore the molecular mechanism underlying the WAY induced ADRP down-regulation, HepG2 cells were treated with PA. PA-induced intracellular triglyceride elevation was reduced by the supplement of WAY in a dose-dependent manner (Fig. 3A). Furthermore, PA increased the transcription of ADRP but did not change lipid droplet (LD) proteins, TIP47 and Perilipin (Fig. 3B). And the induction of ADRP expression was abolished by WAY treatment in a dose-dependent manner (Fig. 3C and D). Moreover, WAY attenuated PA-induced lipid accumulation through specifically suppressing ADRP expression. As shown in Fig. 3E and F, siRNA against ADRP effectively blocked ADRP expression level in Huh7 cells. Triglyceride content was dramatically attenuated by ADRP depletion. But WAY treatment didn't further affect TG level after ADRP depletion, suggesting that the activation of FXR inhibits lipid accumulation through repressing ADRP expression.

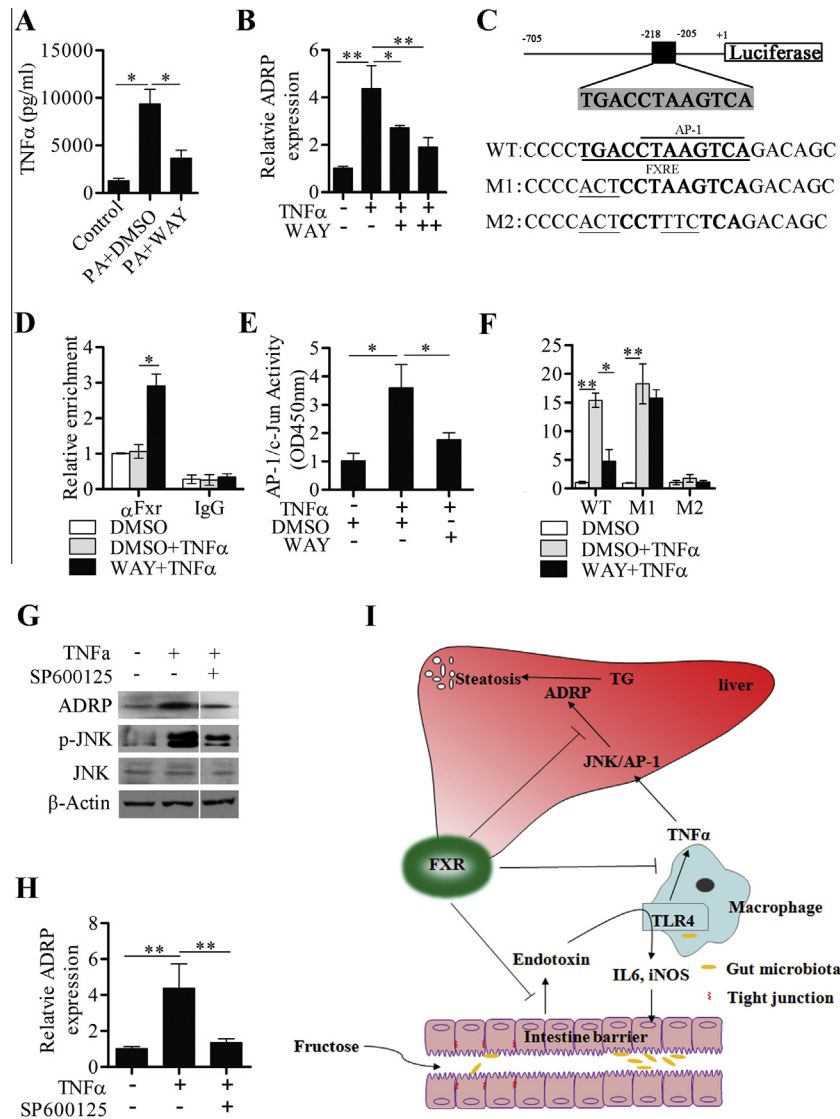


Fig. 4. WAY-362450 inhibits TNFα induced ADRP expression via blocking AP-1 binding to ADRP promoter. (A) TNFα level in cell-free culture supernatants were determined by ELISA in Huh7 cells. (B) Quantitative of relative ADRP mRNA level by qRT-PCR in Huh7 cells. (C) The FXR response element and AP-1 binding site are shown in bold letters and underlined letter as indicated. Site mutations in M1 and M2 mutants are indicated with underlined letters. (D) qChIP analysis was employed to demonstrate FXR binding activity on the ADRP promoter induced by TNFα with or without WAY treatment. (E) WAY abolished TNFα-induced AP-1/c-Jun activity determined by ELISA. (F) Relative promoters' activity of the wild type and the mutants induced by TNFα with or without WAY treatment. (G) JNK inhibitor SP600125 (10 μM) effectively inhibited JNK activity indicated by JNK phosphorylation induced by TNFα. (H) SP600125 completely abolished the up-regulation of ADRP by TNFα. (I) A model illustrating the mechanisms involved in the protective effect of WAY against fructose induced NAFLD. Error bars represent SEM. **p* < 0.05, ***p* < 0.01.

3.4. WAY-362450 inhibited TNFα induced ADRP expression via blocking AP-1 binding site on the ADRP promoter

Then we explored how WAY, an agonist of nuclear receptor FXR, regulates ADRP transcription. As shown in Fig. 4A and B, WAY treatment abolished PA-induced TNFα production, and suppressed TNFα-induced up-regulation of ADRP in a dose-dependent manner. We then analyzed the human ADRP promoter within 5'-UTR region by TFSEARCH. As shown in Fig. 4C, we identified a putative FXR response element (FXRE) site in the ADRP promoter (−218/−205 bp), which is highly conserved across species. Also we discovered a conserved AP-1 binding element within the putative FXRE. We then evaluated the recruiting of activated FXR to the FXRE with qChIP assay and demonstrated that WAY promoted the binding of FXR to the ADRP promoter (Fig. 4D).

Next we examined whether TNFα-induced ADRP transcription is mediated by stimulating AP-1 binding to the Ets/AP-1 element.

AP-1/c-Jun activity was evaluated in TNFα-stimulated HepG2 cells pretreated with or without WAY. As shown in Fig. 4E, WAY attenuated the AP-1 binding activity induced by TNFα. To further elucidate the blocking effects of WAY on TNFα-induced ADRP expression, two ADRP promoter mutants (Fig. 4C) were cloned and tested for their effects on TNFα-induced ADRP expression using a luciferase promoter report assay. As shown in Fig. 4F, M1 mutant, which carries point mutations only within the FXRE region, completely abolished FXR-mediated repression of ADRP promoter activity in the presence of TNFα; while M2 mutant, which carries point mutations within both FXRE and AP-1 binding sites, abolished ADRP promoter activity in the presence of both TNFα and WAY. Taken together, these findings demonstrate that FXR activation inhibits TNFα-induced ADRP expression through competing with AP-1 to bind to the ADRP promoter. Furthermore, we found that TNFα-induced ADRP expression is mediated by JNK. As shown in Fig. 4G and H, a JNK inhibitor SP600125 (10 μM),

which effectively inhibited JNK activity indicated by JNK phosphorylation, completely abolished the up-regulation of ADRP by TNF α .

Therefore, it is evident that WAY restored PA-induced hepatocellular damage through two means, attenuating TNF α production and competing with TNF α -induced JNK-AP-1 binding to Ets/AP-1 element in the ADRP promoter region.

4. Discussion

In the present study we demonstrated that a potent and orally active FXR agonist, WAY, protects against fructose-induced NAFLD via multiple mechanisms. Mice fed 30% fructose showed significant hepatic steatosis, accompanied with increased portal vein endotoxin level, inflammation infiltration, intestine permeability and ADRP expression up-regulation. Activation of FXR by WAY prevents epithelial deterioration, endotoxin translocation, over-activated inflammatory response, and decreases lipid accumulation through suppression of ADRP expression.

Zhang et al. previously found that WAY protects against MCD diet-induced NASH through attenuating hepatic inflammation and fibrosis, but not by reducing hepatic triglyceride accumulation [7]. Whereas, in the present study, in both fructose- and LPS-induced NAFLD mouse model, we observed that WAY reduced TG accumulation. Also, WAY reduced lipid accumulation induced by PA in HepG2 cells. Activation of FXR has been shown to lower hepatic TG levels through regulating genes involved in glucose and lipid metabolism, such as SREBP1c and ChREBP for lipogenesis, AKR1B7 and PDK4 for lipid oxidation, and through increasing plasma lipoprotein clearance, such as ApoC-III and ANGPTL3 [6,17]. Although intravascular lipoprotein catabolism was not directly assessed, our study showed that hepatic gene expression of both fatty acid synthesis and lipid β -oxidation was not altered by WAY treatment (Fig. S1), indicating that it is unlikely for WAY to directly target the expression of *de novo* fatty acid synthesis and lipolysis genes, but rather to down-regulate a specific lipid droplet protein, ADRP. A previous study reported that LPS-induced TNF α elevation caused increased lipolysis in abdominal fat, which in turn led to the influx of fatty acids (FAs) into liver [18]. WAY appeared to lower hepatic TG by reducing TNF α mediated influx of Fas [18] and by inhibiting ADRP mediated storage of lipids.

It has been reported that ADRP antisense oligonucleotide reduced liver steatosis in ob/ob and diet-induced obese mice [19]. LPS-induced liver steatosis was accompanied with increased expression of ADRP [20]. TNF α up-regulated ADRP in differentiated adipocytes [21]. All these observations indicate that up-regulated ADRP expression is a common molecular event mediating lipid accumulation in the liver, regardless of the cause. We found that WAY attenuated TNF α production and competed with TNF α -induced JNK-AP-1 binding to Ets/AP-1 element on ADRP promoter in PA treated hepatoma cell. Therefore, it appears that WAY reduces ADRP expression level through both indirect and direct pathways.

High consumption of fructose increases the risk of developing NAFLD [9]. In the present study, we found that WAY treatment reversed fructose-induced hepatic steatosis through activating FXR. In addition, a previous report also indicated that the activation of FXR protects against bacterial proliferation and its detrimental effects in the distal small intestine through the regulation of Ang1, iNos, and IL18 [22]. It is revealed from this study that WAY mediated enteroprotection is associated with endotoxin translocation, the regulation of Cd68, IL-6, and Claudin-2 expression. However, FXR-independent mechanism also plays a role in protecting liver from hepatic steatosis. Volynets' et al. found that bile acids prevent fructose-induced hepatic steatosis in mice

through blocking fructose-induced translocation of intestinal bacterial endotoxin, whereas hepatic FXR protein concentration did not differ between groups fed with or without fructose [23]. Recently, our group also found that super-physiological concentrations of hepatic bile acids inhibited fatty acid uptake and triglyceride accumulation in FXR-/-/MCD mice [24].

Together with previous reports, we developed a model (Fig. 4I) to illustrate the pathways involved in the protective effect of WAY. Other than the detrimental effects of the metabolites from excess fructose consumption, high fructose intake leads to intestine microbiosis [8,25]. Coupled with intestine barrier disruption, and translocation of endotoxin, microbiosis can subsequently activate macrophages [26]. Activated macrophages secrete more TNF α , which subsequently induces ADRP expression via JNK/AP-1 pathway. Elevation of ADRP promotes lipid accumulation, in turn NAFLD. Activation of FXR blocks endotoxin release and attenuates TNF α production. Moreover, FXR suppresses ADRP transcriptional activity by competing with AP-1 to bind to ADRP promoter. Decreased ADRP expression in turn attenuates fructose induced NAFLD. Taken together, activation of FXR by WAY compromises the “first hit” (lipid accumulation) as well as the “second hit” (endotoxin and inflammatory cytokines) to protect liver function. WAY, as well as other FXR agonists, may represent a new candidate for NAFLD treatment.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2014.05.072>.

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