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Targeted Metabolomics Reveals a Protective Role for Basal PPAR α in Cholestasis Induced by α -Naphthylisothiocyanate

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Abstract

α -Naphthylisothiocyanate (ANIT) is an experimental agent used to induce intrahepatic cholestasis. The *Ppara*-null mouse line is widely employed to explore the physiological and pathological roles of PPAR α . However, little is known about how PPAR α influences the hepatotoxicity of ANIT. In the present study, wild-type and *Ppara*-null mice were orally treated with ANIT to induce cholestasis. The serum metabolome of wild-type mice segregated from that of the *Ppara*-null mice, driven by changes of bile acid (BA) metabolites. Alkaline phosphatase and total BAs were elevated preferentially in *Ppara*-null mice, which correlated with changes in *Cyp7a1*, *Cyp8b1*, *Mrp3*, *Cyp3a11*, *Cyp2b10*, *Ugt1a2*, and *Ugt1a5* genes and showed cross-talk between basal PPAR α and potentially adaptive pathways. *Il6*, *Tnfa*, and target genes in the STAT3 pathway (*Socs3*, *Fga*, *Fgb*, and *Fgg*) were up-regulated in *Ppara*-null mice but not in wild-type mice. The JNK pathway was activated in both mouse lines, while NF- κ B and STAT3 were activated only in *Ppara*-null mice. These data suggest protection against cholestasis by basal PPAR α involves regulation of BA metabolism and inhibition of NF- κ B/STAT3 signaling. Considering studies on the protective effects of both basal and activated PPAR α , caution should be exercised when one attempts to draw conclusions in which the PPAR α is modified by genetic manipulation, fasting, or activation in pharmacological and toxicological studies.

Graphical Abstract

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Supporting Information

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Tables showing a list of the primer sequences used in the Q-PCR analysis in this study and a summary of ions altered. Figures showing the identification of TUDCA, β MCA, TCA, CA, T- α/β / ω MCA, and α/ω MCA by fragmentation profiles and retention times and the expression of PPAR α target-gene mRNAs. (PDF)

The authors declare no competing financial interest.

Keywords

PPAR α ; NF- κ B; STAT3; α -naphthylisothiocyanate; cholestasis

INTRODUCTION

Cholestasis, including primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC), often causes liver injury.¹ In Northern Europe, the PBC prevalence was between 7 and 402 cases per million people.^{2,3} In Asian countries, the PBC prevalence was 400–500 cases per million people.^{4,5} For PSC, the published prevalence was 16.2 per 100 000 people.^{6,7} Cholestasis is still one of the most challenging diseases to be treated in the clinic. In animal models, the pathological and physiological reactions found with α -naphthylisothiocyanate (ANIT)-induced cholestasis are considered to be similar to intrahepatic cholestasis observed in human.⁸ Thus, ANIT is the most widely used experimental chemical to establish animal models for mechanism investigation of cholestatic liver disease.^{9–11}

Peroxisome proliferator-activated receptor α (PPAR α) has a complicated role in modulation of bile acid (BA) homeostasis.¹² In wild-type mice, not the *Ppara*-null mice, gemfibrozil disrupts BA homeostasis by inducing cholesterol 7 α -hydroxylase, resulting in increased tauro- α -muricholic acid/tauro- β -muricholic acid (T- α / β MCA) and taurocholic acid (TCA).¹³ Similarly, when treated with WY-14,643, an increase of gallbladder cholic acid was only observed in wild-type mice.¹⁴ This suggests that PPAR α has a disruptive role on BA homeostasis and hepatotoxicity. However, there were also reports suggesting that PPAR α has a protective role. Notably, an elevation of TCA and cholic acid (CA) was observed in CA-treated *Ppara*-null mice.¹⁵ In another study, pretreatment with fenofibrate protected wild-type mice against ANIT-induced liver toxicity, and this action was negative in *Ppara*-null mice,¹⁶ whereas the role of PPAR α in cholestatic liver disease is not fully understood.

Cholestatic liver injury involves several known inflammation pathways. In bile-duct-ligated mice, NF- κ B was activated, and the toxic phenotype was reversed by an NF- κ B inhibitor.¹⁷ In cholestatic livers from patients and bile duct-ligated rats, the expression and nuclear translocation of p-STAT3 was significantly lower.¹⁸ In an ANIT-treated mouse model, chlorogenic acid inhibited cholestatic liver injury. The protection was associated with an inhibition of NF- κ B and STAT3 phosphorylation.¹⁹ In the HepG2 cell line, the c-Jun N-terminal kinase (JNK) inhibited bile acid synthesis and reduced the toxic effects of pro-inflammatory agents.²⁰ The JNK pathway was found to mediate cholestatic liver injury induced by ANIT, and JNK inhibition significantly attenuated liver injury.¹⁶ Thus, the NF- κ B, STAT3 and JNK pathways were all involved in cholestasis. However, regarding the inflammatory pathways in cholestasis, the involvement of basal PPAR α has not been investigated.

The expression of BA synthesizing enzymes and BA transporters are altered upon cholestasis, including CYP7A1, NTCP, and OATPs, which increase intracellular BA levels, and MRP3, MRP4, BSEP, MRP2, and MDR3, which decrease intracellular BA levels.²¹⁻²³ Besides the adaptive modification by cholestasis, two farnesoid X receptor (FXR)-dependent mechanisms were found to inhibit BA production.²⁴ In the liver, a small heterodimer partner was induced by FXR, which, in turn, inhibits the transcriptional activation of liver receptor homologue-1 and hepatic nuclear factor 4 α , which both regulate *Cyp7a1* and *Cyp8b1* involved in BA synthesis.²⁵ In the intestine, BAs activate FXR and induce fibroblast growth factor 15/19, which increases extracellular-regulated kinase 1/2 and JNK1/2 signaling to decrease expression of the *Cyp7a1* and *Cyp8b1* genes.²⁶ However, the involvement of basal PPAR α in regulating BA metabolism remains elusive.

In the present study, wild-type and *Ppara*-null mice were orally administered with ANIT to induce cholestasis and liver injury. The different toxic responses and adaptation of BA metabolism in wild-type and *Ppara*-null mice were observed. A critical role of basal PPAR α in regulating NF- κ B/STAT3 signaling to protect against inflammation in cholestatic liver injury was revealed.

EXPERIMENTAL PROCEDURES

Materials

α -Naphthylisothiocyanate (ANIT), cholic acid (CA), tauroursodeoxycholic acid (TUDCA), taurodeoxycholic acid (TDCA), tauro- ω -muricholic acid (T ω MCA), tauro- α -muricholic acid (TaMCA), taurocholic acid (TCA), β -muricholic acid (β MCA) and ω -muricholic acid (ω MCA) were purchased from Sigma-Aldrich (St Louis, MO). Assay kits for the liver injury markers aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholestasis markers total bile acid (TBA), alkaline phosphatase (ALP), direct bilirubin (DBIL), and total bilirubin (TBIL) were purchased from Ruiyuan Biotechnology (Ningbo, China). TRIzol solution was purchased from Invitrogen (Dalian, China). The reverse transcription kit and LightCycle 480 SYBR Green I Master Mix were purchased from Roche Diagnostics (Shanghai, China). Antibodies against total mitogen-activated protein kinase kinase 4 (t-MKK4), phospho-mitogen-activated protein kinase kinase 4 (p-MKK4), total-JNK (t-JNK), and phospho-JNK (p-JNK) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against NF- κ B subunit p65, phospho-p65 (p-p65), total STAT3 (t-STAT3), phospho-STAT3 (p-STAT3), and GAPDH were acquired from Abcam.

Animals and Treatments

Animal procedures followed the Institute of Laboratory Animal Resource guidelines, and the protocols were approved by the Animal Care and Use Committee of Ningbo University. Prior to the experiments, 24 to 28 g male 8- to 10-week-old wild-type and *Ppara*-null mice were housed at the Medical School of Ningbo University Animal Center for 7 days of acclimation at 23 ± 1 °C, with a humidity of 60–70%. The mice were provided with standard mouse chow with free access to water under a light/dark cycle of 12 h. A pair of mouse lines were randomly assigned into four groups: vehicle/control (WT-C), ANIT/control (WT-A), vehicle/control (KO-C), and ANIT/control (KO-A). ANIT (75 mg·kg⁻¹ in corn oil) was orally administered to the indicated groups, and 48 h later, the mice were weighed and killed using carbon dioxide asphyxiation. Blood, liver tissues, and gallbladder tissues were collected and scaled to calculate liver and gallbladder indexes. Liver sections were cut and fixed in 10% formalin solution, and the remainders were immediately frozen on dry ice and stored at -80 °C.

Metabolomic Analysis of Serum and Biomarker Identification

Processing of serum samples followed previously published procedures,²⁷ using a different detection system. Specifically, 2 μ L of the supernatant was injected into a nanoultra-performance liquid chromatography-coupled quadrupole time-of-flight mass spectrometer (nano-UPLC Q-TOF MS/MS ACQUITY UPLC, Waters). The ion source of the nano-UPLC was switched to an ESI and tubes of a bigger diameter were used in which a flow rate of 30–60 μ L/min could be tolerated. This modified system produced high pressure and sufficient performance as the normal UPLC-TOF system. A reverse-phase column (ACQUITY UPLC HSS T3, 1.7 μ m 1.0 $\times$ 100 mm) was utilized for chromatograph separation. The mobile phase was set at 35 μ L/min, which started from 100% A for 0.5 min, then increased to 100% B over the next 7.5 min and finally returned to 100% A within the last 2 min (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile).

In the ion source, the source temperature was set at 100 °C with a cone gas flow of 10 L/h, and the desolvation temperature was set at 400 °C with a desolvation gas flow of 400 L/h. The cone voltage and the capillary voltage were set at 30 V and 0.3 kV, respectively. To facilitate the identification of biomarkers, the mass spectra were collected simultaneously in centroid mode using the MS^E function (low energy: 4 eV; high-energy ramp: 20–35 eV; *m/z* range of 100–800; scan time: 0.1 s). During the analysis, leucine enkephalin (0.2 ng/ μ L), dissolved in acetonitrile/water (50:50, v/v) containing 0.1% formic acid, was used as a lock-mass solution. It was infused into the detection system with a flow of 5 μ L/min to ensure accurate masses (± 2 ppm) obtained in each run.

Multivariate Data Analysis

Multivariate data analysis was performed as published procedures with minor modifications.²⁷ After being processed by Marker Lynx, a data matrix of peak areas organized by retention time and *m/z* was generated and then exported into SIMCA 13.0.3 (Umetrics, Kinnelon, NJ) for pareto transformation. Then unsupervised principal component analysis (PCA) was used to produce score plots. Orthogonal projection to latent structures discriminant analysis (OPLS-DA) was exploited to produce the loading S-plot of ANIT-treated mice versus the control mice, in which the BAs contributing to the pattern recognition were exhibited. Accurate molecular weights of known BA components were used to match the contributing items, which were determined to be TUDCA, TDCA, β MCA, TCA, CA, α/ω MCA, and T- $\alpha/\beta/\omega$ MCA by subsequent comparison of the tandem mass spectrometry (MS/MS) spectra with those of the authentic compounds. The relative abundance of these BAs in the serum were normalized by comparing their peak areas with those of internal standard. Fold changes versus the wild-type control group were used to express their systemic abundance in the ANIT-treated mice.

Biochemical Analysis and Histopathological Assessment

TBA, ALP, ALT, and AST in serum were assayed by the Thermo Scientific Multiskan GO. The measurements were carried out following protocols supplied with the kits. Formalin-fixed liver tissues were dehydrated in alcohol and xylene following the standard procedures and embedded by paraffin for preparation of four-micrometer sections. After staining with hematoxylin and eosin, the liver sections were examined under a microscope. A total of 10 sections per preparation were imaged, and all images were analyzed blindly.

Messenger RNA Quantification

The total RNA extraction, the reverse transcription of cDNA, the amplification program were the same as established procedures.¹⁹ The primer sequences extracted from a public database (<http://mouseprimerdepot.nci.nih.gov>) were used in this study (Table S1). A 5 μ L PCR system was designed for the 384 plate that included 1 μ L of total cDNA, 2.5 μ L of LightCycle 480 SYBR Green I Master Mix, 0.2 μ L of forward and reverse primer, and 2 μ L of nuclease-free water.

Western Blot Analysis

Freshly cut liver tissues were transferred to RIPA buffer by 1:10 (g/v) with 1% PMSF and homogenized using MagNA Lyser (Roche). The protein concentrations were determined using a Beyotime BCA kit (Nantong, China). The same volume of 2 X loading buffer was mixed with the samples that were subject to boiling for 5 min. After separation on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the samples were blotted onto PVDF membranes followed by 4 h of blocking with 5% fat-free milk at room temperature. Then the membranes were incubated overnight with primary antibody solutions. After incubation with secondary antibody for another 1.5 h, the membranes were exposed to Western-Bright ECL (Advansta), and the images were collected using Tanon 4200SF (Shanghai, China).

Statistical Analysis

All of the data are presented as mean $\pm$ SD and analyzed independently by two coauthors to ensure the correct conclusions. The investigators treating the mice were not aware of the pharmacological treatments of each group by numbering during the scoring and thereafter accessed to the administration sheet during the data analysis. The data analysis was performed using SPSS 23 (IBM). Differences among the treatment and control groups were tested with one-way ANOVA followed by Dunnett's post-hoc comparisons. Significant difference was decided when $P < 0.05$ and was marked with asterisks in the graphs accordingly.

RESULTS

Serum Metabolome in Wild-Type and *Ppara*-Null Mice

Unsupervised PCA analysis of the four groups revealed that serum samples of the two control mouse lines grouped together. As expected, samples from the two groups treated with ANIT clustered away from the two control groups. However, they moved in different directions, suggesting different modifications of the serum metabolome were induced by ANIT challenge in the two mouse lines (Figure 1A).

The contributing items in the loading S-plots of ANIT-treated mice versus the control mice were visualized in Figure 1B,C. The metabolic biomarkers with VIP values higher than 1.0 were listed in Table S2. A total of seven of the contributing ions were identified as TUDCA, β MCA, TCA, CA, α/ω MCA, and T- $\alpha/\beta/\omega$ MCA by comparison with authentic standards. The identification of TDCA was shown in Figure 1D-G, and the identification of the other six BA components was shown in Figures S1-S6. The approximate abundance of TUDCA and TDCA was 5- and 8-fold higher in wild-type mice than those of the control mice. In contrast, they were only increased 3-fold in *Ppara*-null mice (Figure 2A-C). The TCA, CA, α/ω MCA, and T- $\alpha/\beta/\omega$ MCA were similarly increased by ANIT challenge in the two different mouse lines except that β MCA was increased significantly in KO-A group in comparison with that of WT-A group (Figure 2D-H).

Phenotype Difference in Wild-Type and *Ppara*-Null Mice

The serum ALP, TBA, ALT, and AST were significantly increased in two mouse lines treated with ANIT ($P < 0.05$, Figure 3A), while the ALP and TBA increased more in the KO-A group compared with the WT-A group ($P < 0.05$, Figure 3A). These data indicated that liver injury might be associated with the TBA levels and BA components in the two mice lines. Pathological analysis of the hepatic tissues in both the WT-C and KO-C groups exhibited normal histology (Figure 3A). The WT-A group displayed a disappearance of cellular boundaries, marked degeneration and necrosis (Figure 3B). However, in addition to the above changes, congestion and edema were observed in the KO-A group (Figure 3B). These data indicated that the toxic response differed between wild-type and *Ppara*-null mice.

Alteration of mRNAs Encoding Bile-Acid Metabolism and Transport Proteins

The *Cyp7a1* and *Cyp8b1* mRNA levels in the WT-A groups were decreased by 97% and 94% compared with the WT-C group, respectively ($P < 0.05$). In the KO-A mice, they were almost totally inhibited (100% and 93%) compared with the KO-C group ($P < 0.05$, Figure 4A,B). Thus, the response of CYP7A1 was more pronounced in *Ppara*-null mice. By Western blot analysis, both CYP7A1 and CYP8B1 proteins were decreased in the two cholestatic groups, but CYP7A1 in the WT-C group was also significantly lower than that of the KO-C group. Thus, these two genes appeared to be regulated by both adaptation and basal PPAR α (Figure 4C). *Oatp1* mRNA was decreased by 54% in the WT-A group and 82% in the KO-A group compared with their control groups. The *Oatp2* mRNA level in WT-A was similar to the WT-C group. In contrast, it was decreased by 83% in *Ppara*-null mice ($P < 0.05$, Figure 4D,E). Thus, *Oatp1* and *Oatp2* were regulated through an adaptative mechanism.

The basolateral efflux transporters multidrug-resistance-related protein 3 (MRP3), MRP4, organic solute-transporter- β (OSTB), and BSEP are involved in export of BAs to decrease BA levels in hepatocytes. The *Mrp3* mRNA in WT-A group was increased by 9-fold ($P < 0.05$). However, *Mrp3* mRNA in the KO-A group was only increased by 2.5-fold ($P < 0.05$, Figure 5A). The *Ostb* mRNA in the WT-A group and KO-A group was increased by 204-fold and 225-fold, respectively ($P < 0.05$, Figure 5B). The *Mrp4*, *Bsep*, *Mdr2*, and *Mdr1a* mRNAs in the WT-A group were increased similarly between *Ppara*-null mice and wild-type mice (4–18-fold, Figure 5C-F). The *Mrp3* mRNA level was increased in the WT-A group but only slightly modified in the KO-A group. This indicated that it is regulated by both adaptation and by PPAR α . Thus, among these transporters, MRP3 was regulated by cross-talk between PPAR α and adaptation, but the others were regulated only by adaptation.

CYPs and UDP-glucuronosyltransferases (UGTs) are involved in BA metabolism in liver. In wild-type mice, the *Cyp3a11* mRNA level was increased by 3.9-fold ($P < 0.05$), but no increase was observed in *Ppara*-null mice. Additionally, the *Cyp2b10* mRNA was increased by 6-fold in WT-A group in comparison with that of the *Ppara*-null mice. The mRNA levels of *Ugt1a2* and *Ugt1a5* in wild-type mice were significantly higher than those in the control mice (3.5- and 5.8-fold, respectively; $P < 0.05$; Figure 5G-J). Their increase did not occur in the *Ppara*-null mice either. Accordingly, the detoxification by CYPs and UGTs under BA overload was critically regulated by cross-talk between PPAR α and adaptation pathways.

NF- κ B/STAT3 Pathway Mediation of the Differential Toxicity Between Wild-Type and *Ppara*-Null Mice

Il6 and *Tnfa* mRNAs were not changed in the WT-A group, while they were significantly increased in the KO-A ($P < 0.05$). The *Il10* and *Il1b* mRNAs were slightly increased in the WT-A group compared with the WT-C group (11-fold, 4-fold, $P < 0.05$). However, both were sharply increased in the KO-A group (22-fold, 17-fold, $P < 0.05$). *c-Jun* and *c-Fos* mRNAs were increased by 7.4- and 3.1-fold in the WT-A group and 15- and 44-fold in the KO-A group. These data indicated a higher inflammation level in ANIT-treated *Ppara*-null mice than that of wild-type mice (Figure 6).

STAT3 target gene mRNAs, *Socs3*, *Fga*, *Fgb*, and *Fgg*, were not up-regulated in the wild-type groups. However, their expression were increased by 14-, 15-, 12-, and 26-fold, respectively, in the KO-A group compared with the KO-C group (Figure 7A-D, $P < 0.05$), suggesting the differential activation of STAT3 signaling between wild-type and *Ppara*-null mice. In Western-blot analysis, p-MKK4 and p-JNK proteins were activated two mouse lines, indicating that the JNK pathway played an important role in ANIT-induced liver injury (Figure 7E). p-p65 was not modified in the WT-A group but was increased in the KO-A group. p-STAT3, it was not activated in the WT-A group (Figure 7E) but was also significantly activated in the KO-A group. Combining with the above mRNA analysis, the NF- κ B/STAT3 pathway likely mediates a stronger inflammatory response to cholestatic liver injury in *Ppara*-null mice.

DISCUSSION

The histological and biochemical pathological phenotypes obtained with 75 mg/kg ANIT-induced liver injury is considered the most similar to human intrahepatic cholestatic liver disease and thus is a widely accepted mouse model.⁹ In C57BL/6J mice, 75 mg/kg ANIT induced the elevation of a number of hepatic BAs that were correlated with the transcription level of genes in BA metabolism and inflammation.²⁸ In this study, the ALP and TBA in *Ppara*-null mice increased more than those in wild-type mice, but the increase of less-toxic TUDCA and TDCA in wild-type mice was 5- and 8-fold higher compared with those in *Ppara*-null mice. Similarly, CYP and UGT isoforms associated with the BA metabolism were increased in wild-type mice rather than in *Ppara*-null mice. These modifications contributed to the detoxification of BAs and attenuation of liver injury. In terms of inflammation, JNK signaling was similarly activated in the two mouse lines, but NF- κ B and STAT3 were activated only in *Ppara*-null mice. This could account in part for the more-severe toxic responses in hepatocytes of *Ppara*-null mice. The PPAR α target gene *Cyp4a10*, *Acot1*, and *Ehhadh* mRNAs were not changed, indicating that PPAR α was not significantly activated upon ANIT-induced cholestasis (Figure S7). These data suggest that BA markers correlated with the regulation of up-stream metabolism and the down-stream pro-inflammatory response.

In this study, hepatic mRNAs encoded by genes involved in modulating BA, *Cyp7a1*, *Cyp8b1*, *Oatp1*, and *Oatp2*, were all down-regulated in the cholestatic groups. In HepG2 cells, the clinical fibrate ciprofibrate and the experimental PPAR α agonist Wy-14 643 repressed *Cyp7a1* transcription as well as its activity of the enzyme.²⁹ Endogenous ligands

for PPAR α include certain polyunsaturated fatty acids, while CDCA and other BAs are strong activators of FXR. FXR-dependent mechanisms were considered to be involved in the inhibition of BA synthesis through suppressing CYP7A1. In this study, the expression of CYP7A1 appeared to be suppressed by PPAR α under physiological conditions (Figure 4C). However, the reduction of *Cyp7a1* and *Cyp8b1* in the two cholestatic groups was mainly attributed to strong adaptation, considering their correlation with the TBA levels.

OSTB, MRP4, BSEP, MDR2, and MDR1A1 are encoded by genes involved in decreasing BA levels through transporting BAs in and out of hepatocytes. In this study, their expression was up-regulated in the cholestatic groups, in agreement with a previous study.³⁰ Both human MRP3 and rat MRP3 can transport glycocholic acid and tauroolithocholate 3-sulfate, while TCA is transported only by rat MRP3.³¹ In wild-type and *Ppara*-null mice, clofibrate induced the expression of MRP3 by activating PPAR α .³² In C57BL/6 mice, after perfluorodecanoic acid administration, PPAR α and NRF2 markedly increased *Mrp3* mRNA expression, which suggests a compensatory hepatoprotective response for the liver under cholestasis.³³ In the present study, the increased *Mrp3* mRNA in the WT-A group was much higher than that in the KO-A group, which contributed to reduce the BA burden. Thus, *Mrp3* was regulated more by cross-talk involving basal PPAR α compared to adaptation.^{32,34}

BAs are extensively metabolized into glycine or taurine conjugates in liver tissue, and they also undergo sulfation or glucuronidation. Conjugated BAs are water-soluble and are efficiently renally excreted.²¹ Besides the elimination of xenobiotics, some of the CYP and UGT isoforms also contribute to the metabolism of endogenous compounds including BAs and bilirubin. In male C57BL/6 mice, perfluorodecanoic acid induced CYP3A11 and CYP2B10 by activating PPAR α , contributing to regulation of the BA homeostasis.³⁵ UGT1A3 and UGT2B4 are prominent UGT isoforms for BA glucuronidation in humans.^{36,37} Recently, PPAR α was also found to regulate *CYP3A4*, *CYP1A1*, *CYP11A2*, *CYP12B6*, and *CYP12C8* in human primary hepatocytes.³⁸ In the present study, the *Cyp3a11*, *Cyp2b10*, *Ugt1a2*, and *Ugt1a5* mRNAs were elevated in wild-type mouse liver compared to livers from *Ppara*-null mice. Therefore, the regulation of *Cyp3a11*, *Cyp2b10*, *Ugt1a2*, and *Ugt1a5* mRNAs encoding enzymes involved in detoxification was dependent on cross-talk between basal PPAR α and adaptation pathways.

Cholestatic liver injury correlated with inflammation involving JNK, NF- κ B, and STAT3. The anti-inflammation response associated with activated PPAR α was found to decrease cholestasis.³⁹ In cellular models, the JNK pathway was involved in regulation of BA metabolism.²⁰ In mice, the JNK pathway was activated in ANIT-induced cholestasis, and a JNK inhibitor totally attenuated the liver injury, indicating a critical role for JNK signaling in cholestatic liver injury.¹⁶ In this study, JNK signaling was also similarly activated in both wild-type and *Ppara*-null mice. In human aortic vascular smooth muscle cells, activated PPAR α induced inhibitory I κ B α , which negatively regulated NF- κ B, suggesting a mechanism for PPAR α to decrease cholestasis.^{39,40} In C57BL/6N mice, the ANIT-induced hepatotoxicity was associated with the NF- κ B/IL-6/STAT3 axis and activation of PPAR α the inhibited NF- κ B/IL-6/STAT3 signaling.⁹ However, different modifications between cholestatic wild-type and *Ppara*-null mice and how inflammation pathways contribute to liver injury were not reported. In the present study, NF- κ B and STAT3 were activated only in

Ppara-null mice, in association with inflammatory factors and/or their target genes. Activated NF- κ B increases IL-1, IL-6, and TNF- α levels.⁴¹ STAT3 is activated by a variety of inflammatory cytokines including IL-6. Thus, STAT3 signaling might be initiated by NF- κ B pathway and contributes to hepatotoxicity. Considering the close relationship between NF- κ B and STAT3, NF- κ B-STAT3 signaling likely mediated the liver injury in *Ppara*-null mice. Inhibition of NF- κ B/STAT3 signaling could be the result of regulation by basal PPAR α in wild-type mice.

In summary, the toxic responses of ANIT-induced cholestasis were more pronounced in *Ppara*-null mice compared to those in wild-type samples, suggesting a protective role of basal PPAR α . The BA metabolism was regulated by adaptation and cross-talk involving basal PPAR α . This regulation contributed to the serum metabolome modification, which triggered the differential downstream inflammatory pathways. More importantly, basal PPAR α appeared to inhibit NF- κ B/STAT3 signaling in wild-type mice. Thus, caution should be exercised when one attempts to draw conclusions in which PPAR α is modified by genetic manipulation, fasting, or activation. Considering the species difference of PPAR α , this study warrants additional investigations in PPAR α -humanized mouse models and further studies of the exact mechanism by which PPAR α modulates hepatic functions and toxicity responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ANIT	α -naphthylisothiocyanate
ALP	alkaline phosphatase
TBA	total bile acid
ALT	alanine aminotransferase
AST	aspartate aminotransferase
TUDCA	taurodeoxycholic acid
TUDCA	tauroursodesoxy cholic acid
β MCA	β -muricholic acid
TCA	taurocholic acid
CA	cholic acid

βMCA	β -muricholic acid
ωMCA	ω -muricholic acid
T-αMCA	tauro- α -muricholic acid
T-βMCA	tauro- β -muricholic acid
T-ωMCA	tauro- ω -muricholic acid
TDCA	taurocholic acid
T-ωMCA	tauro- ω -muricholic acid
BSEP	bile-salt export pump
Cyp7a1	cholesterol 7 α -hydroxylase
Cyp8b1	sterol 12 α -hydroxylase
c-Jun	transcription factor AP-1-like
Fga	fibrinogen α chain
Fgb	fibrinogen α chain
Fgg	fibrinogen α chain
MDR2	multidrug-resistance protein 2
MRP3	multidrug-resistance-related protein 3
MRP4	multidrug-resistance-related protein 4
MRP2	multidrug-resistance-related protein 2
NTCP	sodium taurocholate cotransporting polypeptide
OATP1	organic anion-transporting polypeptide 1
OSTB	organic solute-transporter- β
p65	NF- κ B p65 subunit
MKK4	mitogen-activated protein kinase kinase 4
STAT3	signal transducer and activator of transcription 3

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Figure 1.

Targeted metabolomic analysis of serum and biomarker identification in mice treated with ANIT. (A) Scores plot of serum metabolite in ANIT treated wild-type (WT) and *Ppara-null* (KO) mice vs their control groups by PCA. (B) S-plot of OPLS-DA recognized serum metabolome in ANIT-treated WT mice vs WT-C mice, in which the contribution of identified bile acids were indicated. (C) S-plot of OPLS-DA-recognized serum metabolome in ANIT-treated KO mice vs KO-C mice, in which the contribution of identified bile acids were indicated. Each point in panel A represented an individual mouse serum sample, and the points in panel B represented contributing metabolites. The values “t[1]” and “t[2]” represent principal components 1 and 2, respectively. The value “p(corr)[1]” represents the interclass difference, and “p[1]” represents the relative abundance of the ions (PCA, principal component analysis; OPLS-DA, orthogonal projection to latent structures discriminant analysis). (D) Total ion count (TIC) chromatogram of m/z 498.2875 of ANIT-treated mouse serum sample when fragmentation was performed. (E) MS/MS spectra of the peak with retention time 11.16 in WT mouse serum in panel D. (F) TIC chromatogram of authentic TDCA when fragmentation was performed at m/z 498.2875. (G) MS/MS spectra of the peak with retention time of 11.18 in panel F.

Figure 2.

Relative abundance of BA components identified in wild-type and *Ppara*-null mice. (A) The relative abundance TDCA in WT and KO mice 48 h after ANIT treatment. (B) The relative abundance TUDCA in WT and KO mice 48 h after ANIT treatment. (C) The relative abundance β MCA in WT and KO mice on 48 h after ANIT treatment. (D) The relative abundance TCA in WT and KO mice 48 h after ANIT treatment. (E) The relative abundance CA in WT and KO mice 48 h after ANIT treatment. (F) The relative abundance α/ω MCA in WT and KO mice 48 h after ANIT treatment. (G) The relative abundance $t\text{-}\alpha/\beta/\omega$ MCA in WT and KO mice 48 h after ANIT treatment. The data were expressed as mean $\pm$ SD (asterisks indicate a comparison between WT-A and WT-C groups and between KO-A and KO-C groups, respectively, and $P < 0.05$; carets indicate a comparison between WT-A and

KO-A groups; TUDCA, tauroursodeoxycholic acid; T $\alpha/\beta/\omega$ MCA, tauro- $\alpha/\beta/\omega$ - muricholic; TCA, taurocholic acid).

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Figure 3.

Toxic phenotype differed between wild-type and *Ppara*-null mice. (A) Biochemical markers of hepatotoxicity after ANIT treatment. ALP, TBA, ALT, and AST in wild-type and *Ppara*-null mice. (B) Histopathology of hepatotoxicity after ANIT treatment. HE staining of liver tissues in the WT-C, WT-A, KO-C, and KO-A groups. Arrows indicate that the sample exhibited a loss of cellular boundaries, degenerative changes, and marked necrosis. The data were expressed as mean ± SD ($n = 5$; asterisks indicate a comparison between WT-A and WT-C groups and KO-A and KO-C groups; carets indicate a comparison between WT-A and KO-A groups; asterisks with carets indicate $P < 0.05$).

Figure 4.

Expression of genes involved in BA synthesis, uptake, and export. (A) *Cyp7a1* mRNA levels in the wild-type and *Ppara*-null mice. (B) *Cyp8b1* mRNA in wild-type and *Ppara*-null mice. (C) Western-blot analysis of differential CYP7A1 and CYP8B1 signaling mediated by PPAR α . (D) *Oatp1* mRNA levels in the wild-type and *Ppara*-null mice. (E) *Oatp2* mRNA levels in the wild-type and *Ppara*-null mice, respectively. The mRNA levels were measured by Q-PCR and normalized by 18S rRNA. Data were from liver samples collected 48 h after ANIT treatment, and two of five of the liver tissues were randomly selected for Western-blot analysis. Messenger RNAs levels in the vehicle-treated control mice were set as 1 and the results expressed as mean $\pm$ SD ($n = 5$, asterisks indicate a comparison between WT-A and WT-C groups and KO-A and KO-C groups, $P < 0.05$; carets indicate a comparison between WT-A and KO-A groups).

Figure 5.

Differential regulation of genes involved in BA metabolism. (A–F) *Mrp3*, *Ostb*, *Mrp4*, *Bsep*, *Mdr2*, and *Mdr1a* mRNA levels in the wild-type and *Ppara*-null mice. (G–J) Hepatic mRNA levels of CYP and UGT isoforms involved in BA metabolism. Messenger RNA levels in the vehicle-treated control mice were set as 1 and the results expressed as mean $\pm$ SD ($n = 5$, asterisks indicate a comparison between WT-A and KO-A groups, $P < 0.05$; carets indicate a comparison between WT-A and KO-A groups).

Figure 6.

Differentially regulated genes involved in inflammation. (A) *Il6* mRNA levels in the wild-type and *Ppara*-null mice. (B) *Il10* mRNA levels in the wild-type and *Ppara*-null mice. (C) *Tnfa* mRNA levels in the wild-type and *Ppara*-null mice. (D) *Ilb* mRNA levels in the wild-type and *Ppara*-null mice. (E) *cJun* mRNA levels in the wild-type and *Ppara*-null mice. (F) *cFos* mRNA levels in the wild-type and *Ppara*-null mice. The mRNA levels were measured by Q-PCR and normalized by 18S rRNA. mRNA levels in the vehicle-treated control mice were set as 1 and the results expressed as mean $\pm$ SD ($n = 5$, asterisks indicate a comparison between WT-A and WT-C groups and KO-A and KO-C groups; carets indicate a comparison between WT-A and KO-A groups; asterisks and carets indicate $P < 0.05$).

Figure 7.

Differential inflammation associated with NF- κ B/STAT3 signaling. (A–D) STAT3 target gene mRNAs, *Fga*, *Fgb*, *Fgg*, and *Socs3* in the wild-type and *Ppara*-null mice. (E) Western blot of components of the JNK signaling pathway in liver extracts and Western-blot analysis of differential NF- κ B and STAT3 signaling mediated by PPAR α . Data were from liver samples collected 48 h after ANIT treatment, and two out of five of the liver tissues were randomly selected for Western-blot analysis. GAPDH was used as a loading control. The mRNA levels were measured by Q-PCR and normalized by 18S rRNA. mRNA levels in the vehicle-treated control mice were set as 1 and the results expressed as mean $\pm$ SD. For Western-blot analysis, the data were from liver samples collected 5 days after fenofibrate treatment, and two were randomly selected for protein analysis. ($n = 5$, asterisks indicate a comparison between WT-A and WT-C groups and KO-A and KO-C groups; carets indicate a comparison between WT-A and KO-A groups; asterisks and carets indicate $P < 0.05$).