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Impaired bile acid handling and aggravated liver injury in mice expressing a hepatocyte-specific RXR α variant lacking the DNA-Binding Domain

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Abstract

Background/Aims—Retinoid X Receptor α (RXR α) is the principal heterodimerization partner of class II Nuclear Receptors (NRs), and a major regulator of gene expression of numerous hepatic processes, including bile acid (BA) homeostasis through multiple partners. Specific contributions of hepatic RXR α domains in heterodimer function in response to either BA load or ductular cholestasis are not fully characterized.

Methods—Wild-type (WT) mice and mice expressing a hepatocyte-specific RXR α lacking the DNA-Binding-Domain (*hs-RxraDex4*^{-/-}), which retains partial ability to heterodimerize with its partners, were fed a 1% Cholic acid (CA) diet for 5 days, a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet for 3 weeks, or control diet.

Results—Serum ALT (6.5-fold;p<0.05), AST (9.3-fold;p=0.06) and BA (2.8-fold;p<0.05) were increased in CA-fed *hs-RxraDex4*^{-/-} mice compared to CA-fed WT mice, but were equally induced between genotypes by DDC-feeding. CA-feeding elevated total (4.4-fold;p=0.06) and unconjugated (2.2-fold;p<0.02) bilirubin levels in *hs-RxraDex4*^{-/-} mice compared to WT mice, but not in DDC-fed *hs-RxraDex4*^{-/-} mice. Increased necrosis and inflammation was observed in CA-fed, but not in DDC-fed *hs-RxraDex4*^{-/-} mice. Apoptotic markers DR5, CK8, CK18 RNA were increased in CA- and DDC-fed *hs-RxraDex4*^{-/-} mice. Cleaved Caspase3, CK18 and P-JNK protein were elevated in CA-fed but not in DDC-fed *hs-RxraDex4*^{-/-} mice. Induction of Ost β and Cyp2b10 RNA was impaired in CA-fed and DDC-fed *hs-RxraDex4*^{-/-} mice. Surprisingly, DDC-fed *hs-RxraDex4*^{-/-} mice showed attenuated fibrosis compared to DDC-fed WT mice.

Conclusions—These two models of cholestasis identify common and injury-specific roles for RXR α heterodimers and the functional relevance of an intact RXR α -DBD in the hepatocytic adaptive cholestatic response.

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Introduction

Bile acids (BA) are synthesized from cholesterol in the liver with subsequent secretion into bile, after which they enter the lumen of the proximal small intestine. Approximately 95% of BA are reabsorbed in the terminal ileum and efficiently returned to the liver through the portal vein. Synthesis and transport of BA is tightly controlled due to their hepatotoxicity at high doses [1-3]. However, in cholestasis, i.e. an impairment of biliary secretion by pathophysiological processes, BA accumulate within the liver exposing hepatocytes to elevated concentrations of BA leading to liver damage, apoptosis and cell death [2]. Hepatocyte injury results in activation of neighboring liver-resident macrophages-Kupffer cells, as well as recruitment and activation of other inflammatory cells including neutrophils and stellate cells [4]. Under normal conditions the liver activates an orchestrated intrinsic adaptive process to prevent BA accumulation and hepatotoxicity via changes in gene expression that lead to increased BA sinusoidal and canalicular efflux as well as decreased BA biosynthesis and uptake [5, 6]. However these changes are not always sufficient in protecting the liver against the high intrahepatic BA accumulation during cholestasis.

BA are natural ligands and activators of Farnesoid X receptor (FXR) and other NRs including PXR, CAR and VDR [3,7], all belonging to the class II Nuclear Receptor (NR) superfamily. Together these receptors coordinately regulate gene expression involved in BA synthesis, metabolism, conjugation, and transport, as well as enzymes critical for xenobiotic biotransformation collectively serving as a protective adaptive response during high BA levels [7].

RXR α is the common necessary heterodimerization partner of many NRs, including FXR, and as such, serves as a master regulator of numerous liver functions. However, specific contributions of the functional domains of RXR α within these heterodimers have not been identified. The current study delineates a role for the DNA-Binding Domain (DBD) of hepatocyte RXR α in BA homeostasis using Cholic Acid (CA) feeding to elevate hepatic BA levels. Our previous studies showed that mice with hepatocyte-specific deletion for exon4 of RXR α (*hs-Rxra Δ ex4 $^{-/-}$*) express an internally truncated RXR α lacking the DBD [8]. Surprisingly, some RXR α -dependent functions were maintained, while others were not, indicating a gene-by-gene or partner-specific effect for the need of an intact RXR α -DBD. The Ligand binding domain (LBD) including its heterodimerization domains were left intact, therefore the mutated protein retained its ability to respond to ligand and heterodimerize with partners [8]. These mice therefore provide a useful model to study hepatocyte-specific roles for RXR α domains in BA handling and we specified functionality for the RXR α -DBD in two intrahepatic models of cholestasis—short-term CA-feeding and biliary tract obstruction with DDC. In this report we show exaggerated liver injury, inflammation, and cell death in response to CA-feeding in *hs-Rxra Δ ex4 $^{-/-}$* mice, and propose a hepato-protective role of hepatocyte RXR α in conditions of BA overload. In a complementary model of cholestasis, feeding of DDC, some adaptive responses overlapped with those induced by CA while others were unique to this intrahepatic biliary tract obstructive model.

Methods

Animals

Eight week old male *hs-Rxra Δ ex4 $^{-/-}$* mice [9] and wild-type (WT) littermates on a mixed C57Bl/6xDBA2x129SV background were fed a diet containing 1% Cholic acid (Harlan Teklad, Madison, WI, USA) or chow for 5 days, after which livers were harvested. In a separate experiment *hs-Rxra Δ ex4 $^{-/-}$* and WT littermates were fed a 0.1% DDC containing diet or chow for 3 weeks [10]. Mice were maintained in a temperature- and humidity-

controlled environment and provided with water and rodent chow ad lib. Animal protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Serum Biochemistry

Blood was collected by cardiac puncture and serum was analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and bilirubin levels (Cobas Integra 400p; Roche) at the Center of Comparative Medicine at Baylor College of Medicine. Serum bile acid levels were evaluated by colorimetric methods (BioQuant Inc, San Diego, CA) according to manufacturer's protocol.

Histology and Immunohistochemistry

Livers were rapidly isolated and fixated in 10% phosphate buffered formalin. Liver sections were subsequently stained with routine hematoxylin-eosin (performed by the Texas Medical Center Digestive Disease Center). CD45 staining was performed and counted as described previously [11]. Ki-67 and Sirius Red was performed by the Yerkes Pathology core (Emory University) according to standard protocols, and analyzed using Image J [12]

Gene expression analysis

Liver RNA was isolated and gene expression was determined as described before [13]. Primers and probes were obtained from Sigma Genosys. Sequences are available on request. All data were analyzed by Two-Way ANOVA. p-values < 0.05 were considered significant.

Protein analysis

Nuclear and cytosolic fractions were prepared as previously described and western blot analysis performed as before [13].

Primary hepatocyte experiments

Primary mouse hepatocytes were isolated and cultured as described previously [13] and treated with ligands for FXR (50 μ M CDCA, 1 μ M Obeticholic Acid; OCA) and RXR α (1 μ M 9-Cis-Retinoic Acid, 1 μ M LG268) for 24 hrs. OCA and LG268 were kindly provided by Intercept Pharmaceuticals and Ligand Pharmaceuticals respectively.

Results

CA-feeding affects bodyweight and liver weight in *hs-Rxra Δ ex4 $^{-/-}$* mice

Body weight (BW) and liver weight (LW) in 1% CA-fed *hs-Rxra Δ ex4 $^{-/-}$* mice was reduced by 3.2 % and 14% respectively (Fig S1A; p<0.05 vs WT/CA; p<0.05 vs KO/CA), whereas BW and LW of WT mice was not significantly affected. LW/BW ratio was reduced by 8 % in CA-fed *hs-Rxra Δ ex4 $^{-/-}$* mice (p<0.01 vs. KO/CA; Fig S1A), without being affected in WT mice. Thus, the absence of an intact RXR α -DBD in hepatocytes has a significant negative influence on mouse body weight after only 5 days of 1% CA feeding.

CA-feeding aggravates liver injury in *hs-Rxra Δ ex4 $^{-/-}$* mice

Serum ALT (6.5-fold; p<0.05;) and AST (9.3-fold; p=0.06;) levels in CA-fed *hs-Rxra Δ ex4 $^{-/-}$* mice were significantly increased compared to CA-fed WT mice (Table 1). ALP levels were mildly, but equally induced by CA in both genotypes (Table 1), indicating the presence of primarily hepatocyte-related damage as opposed to biliary duct damage. Serum total and indirect bilirubin levels were significantly elevated in CA-fed *hs-Rxra Δ ex4 $^{-/-}$* mice (4.4-fold

and 2.2-fold respectively, $p < 0.05$; Table 1), while serum BA were twice as elevated in *hs-RxraΔex4*^{-/-} mice as WT (329 vs 115 μ M compared to 14 μ M in chow fed mice; Table 1). Histologic staining showed increased necrosis in CA-fed *hs-RxraΔex4*^{-/-} mice, but not in CA-fed WT mice (FigS2). Significantly increased serum LDH levels (3.5-fold; $p < 0.05$; Table 1) in CA-fed *hs-RxraΔex4*^{-/-} mice compared to CA-fed WT mice confirmed this observation, altogether indicating a high BA load caused more severe liver damage in *hs-RxraΔex4*^{-/-} mice.

Increased hepatic inflammation by CA feeding in *hs-RxraΔex4*^{-/-} mice

CA-feeding increased TNF α and IL-1 β RNA 3-4 fold in *hs-RxraΔex4*^{-/-} mice relative to the WT/chow group (Fig1A; $p < 0.05$), with a similar trend for IL-6 and F4/80. CA-feeding induced iNOS expression 10-fold in *hs-RxraΔex4*^{-/-} mice (Fig1A; $p < 0.05$), but not in WT mice. In addition, Ccl5 and Tgfb RNA levels were 5- and 2-fold induced respectively, only in CA-fed *hs-RxraΔex4*^{-/-} mice (Fig 1A). Higher numbers for the pan-leukocyte marker CD45 indicated significantly increased infiltration in the CA-fed *hs-RxraΔex4*^{-/-} mice only ($p < 0.05$; Fig1B). Taken together, livers with hepatocytes that express the RXR α - Δ DBD-variant show more inflammation and damage than those with intact RXR α .

Increased hepatic apoptosis in CA-fed *hs-RxraΔex4*^{-/-} mice

CA-fed WT mice showed increased levels of cleaved Caspase3, cleaved Caspase6 and P-JNK protein, which were elevated to a greater extent in the CA-fed *hs-RxraΔex4*^{-/-} mice (Fig2A), suggesting higher activation of apoptosis pathways in CA-fed *hs-RxraΔex4*^{-/-} mice. Protein levels of other signaling pathways previously associated with BA-induced hepatocyte apoptosis (P-Erk, P-Akt, Ikb α , P-Stat3) were unchanged (data not shown).

Apoptosis in hepatocytes depends mainly upon either Fas- or Death Receptor 5 (DR5)-mediated pathways and BA are known to upregulate DR5 via a JNK-mediated pathway [14]. DR5 RNA was significantly increased in CA-fed *hs-RxraΔex4*^{-/-} mice compared to CA-fed WT mice (9.6 $\pm$ 1.8 and 5.8 $\pm$ 1.8 fold vs WT/chow, resp.; $p < 0.05$; Fig2B), whereas Trail RNA was reduced by 50% in both genotypes (Fig2B). Of note, basal DR5 expression was 2-fold ($p < 0.05$) increased in *hs-RxraΔex4*^{-/-} mouse livers suggesting a predisposition, or priming, for DR5-mediated apoptosis. RNA levels of 2 other markers for BA-induced apoptosis, CK8 and CK18 [15-17], were equally induced in both genotypes. CyclinD1 RNA was induced 8-fold ($p < 0.05$) in both genotypes (Fig 2B), with similar results for Foxm1b (Fig 2B), whereas Ki-67 staining was minimal, indicating proliferation was not a factor in increased liver damage in CA-fed *hs-RxraΔex4*^{-/-} mice (Fig S4).

Impaired BA-handling in *hs-RxraΔex4*^{-/-} mice

Many genes involved in hepatic BA adaptation are highly regulated via BA activation of FXR/RXR α -containing heterodimers. RNA levels of the direct FXR/RXR α target genes Bsep (Abcb11) and SHP were increased (2-3fold ($p < 0.05$; Fig.3A) in CA-fed WT mice but unchanged in CA-fed *hs-RxraΔex4*^{-/-} mice. Ntcp RNA was equally reduced by 80% in both CA-fed genotypes (Fig3A). The FXR/RXR α -regulated basolateral BA exporter complex Ost α / β showed an 18 $\pm$ 3.8-fold induction for Ost β in CA-fed WT mice, with markedly impaired induction (8.2 $\pm$ 4.8-fold) in CA-fed *hs-RxraΔex4*^{-/-} mice (Fig3A). Ost α RNA was 50% reduced in CA-fed *hs-RxraΔex4*^{-/-} mice (FigS2). The residual responsiveness of Ost β expression in *hs-RxraΔex4*^{-/-} mouse liver was further studied in primary mouse hepatocytes treated with ligands for FXR (CDCA and OCA) and RXR α (9-CisRA and LG268), and showed a 30-fold induction by CDCA and 181-fold by OCA in WT hepatocytes. Binding of FXR and RXR α to Bsep and Ost β promoter regions followed expression patterns and was induced in CA-fed WT mice, but not in CA-fed *hs-RxraΔex4*^{-/-} mice (Fig3C). A $\sim$ 50% induction remained for both ligands in *hs-RxraΔex4*^{-/-} hepatocytes

(Fig3B). Treatment with RXR α ligands elicited minor inductions of Ost β RNA in WT cells, but none in *hs-Rxra Δ ex4 $^{-/-}$* hepatocytes, showing an intact RXR α - DBD is required for optimal FXR-mediated BA responsiveness for Ostb. Reduced BA synthesis via downregulation of Cyp7a1 and Cyp8b1 RNA levels in CA-fed WT mice was noted, with equally reduced Cyp7a1 in CA-fed *hs-Rxra Δ ex4 $^{-/-}$* mice, but slightly less for Cyp8b1 (Fig3D). Detoxification of BA is mediated by Phase I and II enzymes. Induction of Cyp2b10 and Cyp3a11 in CA-fed WT mice (6.8 $\pm$ 5.7-fold and 26.8 $\pm$ 18.6-fold resp.; $p < 0.05$; Fig3E) was significantly impaired in CA-fed *hs-Rxra Δ ex4 $^{-/-}$* mice. Ugt1a1 and Sult2a1 expression was unaffected by CA-feeding WT mice but RNA levels were reduced in *hs-Rxra Δ ex4 $^{-/-}$* mice ($p < 0.05$; Fig3E). No changes in hepatobiliary transporters Mrp2, Mrp3 and Mrp4 expression levels were noted by diet, genotype, or their combination (FigS2).

Prolonged exposure to BA can cause fibrosis, and the markers Colla1 and Timp1 were highly induced in CA-fed *hs-Rxra Δ ex4 $^{-/-}$* mice but not in CA-fed WT mice (Fig3F). However no changes in fibrosis were observed by Sirius Red analysis (Fig S4), likely due to the short-term duration of the study. Together these results indicate increased liver damage in *hs-Rxra Δ ex4 $^{-/-}$* mice, induced by impaired BA-handling due to dysregulation of hepatic gene expression by the lack of the DBD of RXR α in hepatocytes.

DDC-induced cholestasis in WT and *hs-Rxra Δ ex4 $^{-/-}$* mice

Susceptibility to aggravated liver injury of *hs-Rxra Δ ex4 $^{-/-}$* mice was determined in a second model of cholestasis with intrahepatic biliary tract obstructive damage from DDC feeding for 3 weeks. Bodyweight, liver weight and LW/BW ratio was reduced in DDC-fed WT mice, but not in DDC-fed *hs-Rxra Δ ex4 $^{-/-}$* mice (FigS1). Serum injury markers ALT, AST, ALP as well as serum BA and LDH levels were equally and markedly induced in DDC-fed WT and *hs-Rxra Δ ex4 $^{-/-}$* mice (Table 2). Surprisingly, serum bilirubin induction was impaired in DDC-fed *hs-Rxra Δ ex4 $^{-/-}$* mice compared to WT mice. Despite an equal substantial elevation of serum BA ($> 480 \mu\text{M}$) in both DDC-fed WT and *hs-Rxra Δ ex4 $^{-/-}$* mice, serum bilirubin was predominantly “direct” in WT DDC-fed mice (3.5 mg/dl vs 4.9 mg/dl of total bilirubin level), while total bilirubin levels in DDC-fed *hs-Rxra Δ ex4 $^{-/-}$* mice only reached 0.67 mg/dl (Table 2). Thus, there is a discrepancy from the lack of a hepatocytic RXR α -DBD with respect to bilirubin and BA homeostasis in the setting of substantial hepatocellular damage in the DDC model. Although there were no significant changes in the expression of inflammatory markers Tnf α and iNOS in DDC-fed mice (FigS3), apoptosis markers DR5, CK8 and CK18 were significantly increased in DDC-fed *hs-Rxra Δ ex4 $^{-/-}$* mice, indicating increased apoptosis, but not in DDC-fed WT mice (Fig4A). CyclinD1 RNA showed a similar pattern, with Ki-67 staining confirming increased proliferation in WT-DDC mice but not in DDC-fed *hs-Rxra Δ ex4 $^{-/-}$* mice (FigS4). No changes in P-JNK, cleaved caspase3 or cleaved CK18 were observed (data not shown).

In contrast to CA-feeding, RNA levels of Bsep were downregulated in both DDC-fed WT and *hs-Rxra Δ ex4 $^{-/-}$* mice. The induction of Ost β in DDC-fed WT mice was impaired in *hs-Rxra Δ ex4 $^{-/-}$* mice. Cyp7a1 RNA was markedly reduced in DDC-fed WT mice, with a more modest reduction in *hs-Rxra Δ ex4 $^{-/-}$* mice (Fig4B). ChIP-QPCR analysis for FXR and RXR binding on the Bsep and Ost β promoters did not fully reflect the changes in Bsep RNA expression (Fig4C). These results may indicate the involvement of additional factors at play that distinguish the adaptive responses of a biliary obstructive model (DDC) from that of CA-feeding. Despite equal induction of the fibrosis markers Colla1 and Timp1 by DDC in both genotypes (Fig4D), fibrosis was reduced in DDC-fed *hs-Rxra Δ ex4 $^{-/-}$* mice (FigS4). Taken together, there were many commonalities in the roles for the RXR α -DBD in the DDC and CA-fed models, but point to additional, currently unidentified, contributors from injured cholangiocytes that signal towards hepatocytic adaptations involving an intact RXR α .

Discussion

The current study shows a differential role of the RXR-DBD in the hepatic adaptive responses to liver injury due to high levels of dietary CA or to biliary obstruction due to DDC-feeding. Our previous studies [8] showed gene-specific residual function of RXR α - Δ DBD under inflammatory conditions, likely due to activity through differential interactions of the internally truncated protein with its partners. This prompted us to delineate the residual ability of RXR α -partners to be activated, with in the current study a specific focus on NR-activation by BA and the subsequent physiological adaptation. Overall, liver injury in both models was accompanied by markedly elevated serum biochemistry markers (ALT, AST, bilirubin), increased serum BA levels, elevated proinflammatory, pro-fibrotic and pro-apoptotic responses, along with evidence of increased necrosis (histology and LDH), and finally an impaired hepatic adaptive gene expression responses, showing the RXR α -DBD in heterodimeric NR complexes is required. A significant distinction was observed in the DDC-model: elevations in liver biochemistry markers were equal between genotypes with the interesting exception of serum bilirubin, which was not elevated in DDC-fed *hs-Rxra Δ ex4*^{-/-} mice. This finding is intriguing, and to date not fully explained by the adaptive response genes or intermediaries in cell signaling results from our studies. These findings do point towards discrepant homeostatic mechanisms for direct bilirubin and BA overload in mice with hepatocytes expressing an intact or a DBD-deficient RXR α .

Induction of Ost β and Cyp2b10 in *hs-Rxra Δ ex4*^{-/-} mice was impaired in both CA and DDC cholestatic models, suggesting similar adaptive responses. While basal Ost β and Cyp2b10 RNA levels were 70% lower in chow-fed *hs-Rxra Δ ex4*^{-/-} mice, confirming previous studies [8], RNA levels could be partially induced by CA and DDC-feeding in *hs-Rxra Δ ex4*^{-/-} mice. This residual induction was confirmed in primary mouse hepatocytes treated with FXR ligands, but no induction by RXR α ligands was noted, indicating the remaining functionality of the RXR α - Δ DBD protein occurred most likely via its heterodimerization with FXR. In contrast to CA-feeding, DDC-induced cholestasis reduced Bsep RNA in both genotypes, suggesting a possible additional role for cholangiocyte-derived signaling in regulation of Bsep expression.

A contributing to the observed aggravated hepatic injury in CA-fed *hs-Rxra Δ ex4*^{-/-} mice is the enhanced pro-inflammatory response (TNF α , iNOS, IL-1 β , Ccl5). Whether this is due to direct or indirect roles for RXR α and/or BA needs further study. The reported anti-inflammatory actions of BA through activation of FXR [18], as partner of RXR α , could potentially indirectly contribute to enhanced inflammatory response, due to possible impaired FXR function. BA have also been shown to activate JNK, which is upstream of cytokine activation [2], suggesting the increased phospho-JNK levels in CA-fed *hs-Rxra Δ ex4*^{-/-} mice, could cause the increased inflammatory response. Furthermore, prolonged activation of JNK promotes apoptosis, while increased BA levels enhance JNK-mediated activation of DR5 leading to apoptosis in hepatocytes, suggesting the observed increase in DR5 RNA in CA-fed *hs-Rxra Δ ex4*^{-/-} mice could sensitize the liver to Trail-mediated cytotoxicity [14, 19, 20].

Fibrosis is another adaptive response to cholestatic liver injury and despite increased fibrosis markers in CA-fed *hs-Rxra Δ ex4*^{-/-} mice, fibrosis was not observed likely due to the short-term feeding regiment. In contrast, despite equal induction of Col1a1 and Timp1 RNA levels, interestingly fibrosis was impaired in DDC-fed *hs-Rxra Δ ex4*^{-/-} mice, suggesting the inhibitory effect may occur at the level of collagen deposition.

Multiple other factors are involved in fibrogenesis, and in progression and resolving of fibrosis [21]. Aside from activated hepatic stellate cells, roles for macrophages NK cells

both in progression and repair have been shown to play a role. Several other studies have shown involvement of RXR α and precursors for its ligand (vitamin A and 9-Cis RA respectively) in fibrosis though with conflicting results. Some studies have shown activation of HSC correlates with diminished RXR expression in HSC as well as the reverse [22]. Consistent with a role for RXR, RXR-partners such as PPAR δ , LXR α/β VDR [23-26], are involved in fibrosis. Interestingly however, this was shown in stellate cells, whereas in our model RXR α in hepatocytes is affected, suggesting intercellular communication between hepatocytes, cholangiocytes, macrophages and hepatic stellate cells is of major importance in fibrogenesis. Roles for hepatocyte-produced factors such as CTGF, a hepatocyte produced fibrogenic master switch[27], and hepatocytes Snail1 have been shown to have key roles in fibrosis[28]. If RXR α affects regulation of these genes remains to be determined.

Since RXR α is the obligate partner for several NRs functioning as BA-activated receptors (FXR, CAR, PXR and VDR) it could very well be considered that RXR α is a silent partner in the heterodimeric complex. However hepatic response individual knockout mice for these BA-activated NRs respond differently compared to the CA-fed *hs-Rxra* Δ *ex4* $^{-/-}$ [29-32]. It should also be considered that these were whole mouse knockout models, whereas in our study the DBD of RXR α was specifically deleted in hepatocytes, and may therefore serve as a more specific model for studying roles played by fully-functional RXR α heterodimers.

Previous studies treating *hs-Rxra* Δ *ex4* $^{-/-}$ mice with the ligand for another partner of RXR α , PPAR α , indicated resistance to ligand-induced proliferation and cholestasis [9, 33, 34]. Interestingly, and in contrast to our results, serum ALT and ALP, and serum BA levels were not induced in *hs-Rxra* Δ *ex4* $^{-/-}$ mice [34]. Differential responses of Cyp3a11, CyclinD1 and IL-1 β RNA between those studies and ours indicates the role of DNA binding of RXR α for a particular gene may depend on partner and ligand, as well as the likely altered milieu of the nucleoplasm with respect to the array of potential agonistic and antagonistic ligands for NRs. Future metabolomic studies focused upon this likely feature will help investigate roles played by various altered NR ligands and the need for an intact RXR α -DBD.

In conclusion, we show aggravated liver injury in *hs-Rxra* Δ *ex4* $^{-/-}$ mice after high BA load, indicating the DBD of RXR α is required for appropriate heterodimer activity related to BA homeostasis and non-NR pathways are unable to handle a high BA load. We suggest the mechanisms behind these impaired BA adaptations in *hs-Rxra* Δ *ex4* $^{-/-}$ mice were primarily due to failure to sufficiently activate NR target genes involved in export and detoxification of BA, resulting in accumulation of cytotoxic BAs in liver, leading to engagement of intracellular signaling and necrosis/apoptosis pathways. Moreover, greater injury was likely caused by an enhanced inflammatory response in the *hs-Rxra* Δ *ex4* $^{-/-}$ mice. Hepatocyte RXR α appears to play a major hepato-protective role in BA mediated liver injury but pro-fibrotic role in DDC-mediated liver injury and the data in these uniquely informative models provide insight into the complex and various targeting of RXR α heterodimers in the hepatic adaptive response to cholestasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

RXRα	Retinoid X Receptor α
NR	Nuclear Receptors
DBD	DNA-Binding Domain
CA	cholic acid
BA	bile acids

Figure 1. Elevated hepatic inflammation in Cholic Acid-fed *hs-RxraΔex4*^{-/-} mice

Elevated hepatic gene (expression of pro-inflammatory genes in CA-fed *hs-RxraΔex4*^{-/-} mice as analyzed by real-time PCR (A). Quantification of hepatic parenchyma stained by immunohistochemistry for CD45 positive cells. Values denote volume fraction (% Vol) of cells of interest per hepatic parenchyma per high-power field (60Xoriginal). A total of 10 high-power fields counted per liver per mouse (B). Values are expressed as average±SD obtained from 5-7 animals/group. *p<0.05 vs WT/chow, #p<vs WT/CA, ^p< vs KO/chow.

Figure 2. Increased hepatic apoptosis in Cholic Acid fed *hs-RxraΔex4*^{-/-} mice

Immunoblot analyses show increased levels of P-JNK, cleaved caspases3 and -6 and cleaved cytokeratin18 in cytosolic fractions of livers of CA-fed *hs-RxraΔex4*^{-/-} mice. Total JNK serves as loading control (A). Hepatic gene expression of pro-apoptosis markers DR5, Trail, CK8, CK18 and proliferation markers CyclinD1 and Foxm1b in CA-fed *hs-RxraΔex4*^{-/-} mice (B). Values are expressed as average $\pm$ SD obtained from 5-7 animals/group. * $p < 0.05$ vs WT/chow, # $p < 0.05$ vs WT/CA, ^ $p < 0.05$ vs KO/chow.

Figure 3. Impaired regulation of gene expression involved in hepatic BA handling in Cholic Acid-fed *hs-RxraΔex4*^{-/-} mice

Impaired regulation of genes involved in hepatic BA transport in CA-fed *hs-RxraΔex4*^{-/-} mice (A) or in primary hepatocytes (B). Hepatic FXR and RXRα binding to Bsep and Ostβ promoter regions in CA-fed *hs-RxraΔex4*^{-/-} mice (C). Impaired regulation of genes involved in hepatic BA synthesis (D) and BA detoxification in CA-fed *hs-RxraΔex4*^{-/-} mice (E). Values are expressed as average $\pm$ SD obtained from 5-7 animals/group. * p < 0.05 vs WT/chow, # p < vs WT/CA, ^ p < vs KO/chow.

Figure 4. Gene expression involved in hepatic apoptosis and bile acid handling in DDC-fed *hs-RxraΔex4*^{-/-} mice

Hepatic gene expression of pro-apoptosis markers DR5, CK8, CK18 and proliferation markers CyclinD1, in CA-fed *hs-RxraΔex4*^{-/-} mice (A). Impaired hepatic gene expression of NR-regulated genes involved in hepatic BA transport and metabolism in CA-fed *hs-RxraΔex4*^{-/-} mice (B). Hepatic FXR and RXRα binding to Bsep and Ostβ promoter regions in CA-fed *hs-RxraΔex4*^{-/-} mice (C) and fibrosis markers in CA-fed *hs-RxraΔex4*^{-/-} mice (D). Values are expressed as average $\pm$ SD obtained from 5-7 animals/group. *p<0.05 vs WT/chow, #p<vs WT/CA, ^p< vs KO/chow.

Table 1
Serum liver injury markers in Cholic Acid-fed WT and *hs-Rxra* Δ *ex4*^{-/-} mice

Values are expressed as average $\pm$ SD obtained from 5-7 animals/group. *p< 0.05 vs WT/chow, #p<vs WT/CA, ^p< vs KO/chow.

Serum marker	Chow diet 5 days		1% CA diet 5 days	
	Wt	<i>hs-Rxra</i> Δ <i>ex4</i> ^{-/-}	Wt	<i>hs-Rxra</i> Δ <i>ex4</i> ^{-/-}
ALT (U/L)	13 $\pm$ 5	24 $\pm$ 3*	116 $\pm$ 56*	762 $\pm$ 605*^#
AST (U/L)	50 $\pm$ 11	82 $\pm$ 26*	153 $\pm$ 32*	1430 $\pm$ 1626*
ALP (U/L)	87 $\pm$ 7	71 $\pm$ 16	115 $\pm$ 14 *	118 $\pm$ 19*^
LDH (U/L)	212 $\pm$ 87	497 $\pm$ 154 *	329 $\pm$ 127	1176 $\pm$ 726*^
total bilirubin (mg/dL)	0.08 $\pm$ 0.03	0.14 $\pm$ 0.12	0.13 $\pm$ 0.07	0.62 $\pm$ 0.621
bilirubin direct (mg/dL)	0.02 $\pm$ 0.00	0.05 $\pm$ 0.02*	0.08 $\pm$ 0.03*	0.25 $\pm$ 0.29
indirect bilirubin (mg/dL)	0.10 $\pm$ 0.06	0.10 $\pm$ 0.08	0.07 $\pm$ 0.05	0.22 $\pm$ 0.13*
Bile acids (μ mol/L)	14 $\pm$ 1	14 $\pm$ 5	116 $\pm$ 51*	329 $\pm$ 255 *#

Table 2
Serum liver injury markers in DDC-fed WT and *hs-RxraΔex4*^{-/-} mice

Values are expressed as average $\pm$ SD obtained from 5-7 animals/group. *p< 0.05 vs WT/chow, #p<vs WT/DDC, ^p< vs KO/chow.

Serum marker	Chow diet 3 weeks		0.1% DDC diet 3 weeks	
	Wt	<i>hs-RxraΔex4</i> ^{-/-}	Wt	<i>hs-RxraΔex4</i> ^{-/-}
ALT (U/L)	31 $\pm$ 14	73 $\pm$ 51	1321 $\pm$ 826*	1044 $\pm$ 463*^
AST (U/L)	84 $\pm$ 47	109.13 $\pm$ 50.28	1285.44 $\pm$ 788.12*	965.09 $\pm$ 399*^
ALP (U/L)	109 $\pm$ 9	135.38 $\pm$ 106.71	335 $\pm$ 83.41*	400 $\pm$ 157*^
LDH (U/L)	319 $\pm$ 149	328.25 $\pm$ 156.65	1608.11 $\pm$ 923.08*	1403.64 $\pm$ 585.07*^
total bilirubin (mg/dL)	0.15 $\pm$ 0.032	0.3 $\pm$ 0.11	4.94 $\pm$ 2.11*	0.67 $\pm$ 0.25^#
bilirubin direct (mg/dL)	0.04 $\pm$ 0.0098	0.1 $\pm$ 0.047*	3.51 $\pm$ 1.50*	0.21 $\pm$ 0.087#
indirect bilirubin (mg/dL)	0.1 $\pm$ 1.50 E-17	0.22 $\pm$ 0.10*	1.42 $\pm$ 0.64	0.45 $\pm$ 0.16
Bile acids (μ mol/L)	14 $\pm$ 2	37 $\pm$ 23	498 $\pm$ 693*	486 $\pm$ 287*^