Research Article

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Impaired bile acid handling and aggravated liver injury in mice expressing a hepatocyte-specific RXRα variant lacking the DNA-binding domain

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Background & Aims: Retinoid X Receptor α (RXRα) 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 RXRα 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 RXRα lacking the DNA-Binding-Domain (hs-*Rxrα*Δex4$^{-/-}$), 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-*Rxrα*Δex4$^{-/-}$ 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-*Rxrα*Δex4$^{-/-}$ mice compared to WT mice, but not in DDC-fed hs-*Rxrα*Δex4$^{-/-}$ mice. Increased necrosis and inflammation was observed in CA-fed, but not in DDC-fed hs-*Rxrα*Δex4$^{-/-}$ mice. Apoptotic markers *DR5*, *CK8*, *CK18* RNA were increased in CA- and DDC-fed hs-*Rxrα*Δex4$^{-/-}$ mice. Cleaved caspase 3, CK18 and p-JNK protein were elevated in CA-fed but not in DDC-fed hs-*Rxrα*Δex4$^{-/-}$ mice. Induction of *Ostβ* and *Cyp2b10* RNA was impaired in CA-fed and DDC-fed hs-*Rxrα*Δex4$^{-/-}$ mice. Surprisingly, DDC-fed hs-*Rxrα*Δex4$^{-/-}$ mice showed attenuated fibrosis compared to DDC-fed WT mice.

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

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 and thereby 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].

Keywords: Bile acids; Liver injury; Nuclear receptors; Retinoid x receptor; Apoptosis; DDC; Biliary fibrosis.
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Abbreviations: RXRα, Retinoid X Receptor α; NR, Nuclear Receptors; DBD, DNA-Binding Domain; CA, cholic acid; BA, bile acids.

RXRα 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 RXRα within these heterodimers have not been identified. The current study delineates a role for the DNA-Binding Domain (DBD) of hepatocyte RXRα 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 RXRα (hs-*Rxrα*Δex4$^{-/-}$) express an internally truncated RXRα lacking the DBD [8]. Surprisingly, some RXRα-dependent functions were maintained, while others were not, indicating a gene-by-gene or partner-specific effect for the need of an intact RXRα-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 RXRα domains in BA handling and we specified functionality for the RXRα-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-*Rxrα*Δex4$^{-/-}$ mice, and propose a hepato-protective role of hepatocyte RXRα 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.

Materials and methods

Animals

Eight week old male hs-*Rxrα*Δex4$^{-/-}$ 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-*Rxrα*Δex4$^{-/-}$ 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 400þ; 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 μM CDCA, 1 μM Obeticholic acid; OCA) and RXRα (1 μM 9-Cis-Retinoic acid, 1 μM LG268) for 24 h. OCA and LG268 were kindly provided by Intercept Pharmaceuticals and Ligand Pharmaceuticals respectively.

Results

CA-feeding affects bodyweight and liver weight in hs-Rxrα⊿ex4$^{-/-}$ mice

Body weight (BW) and liver weight (LW) in 1% CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice was reduced by 3.2% and 14% respectively (Supplementary Fig. 1A; 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-*Rxrα*Δex4$^{-/-}$ mice (p <0.01 *vs.* KO/CA; Supplementary Fig. 1A), without being affected in WT mice. Thus, the absence of an intact RXRα-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-Rxrα⊿ex4$^{-/-}$ mice

Serum ALT (6.5-fold; p <0.05) and AST (9.3-fold; p = 0.06) levels in CA-fed hs-*Rxrα*Δex4$^{-/-}$ 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-*Rxrα*Δex4$^{-/-}$ mice (4.4-fold and 2.2-fold, respectively, p <0.05; Table 1), while serum BA were twice as elevated in hs-*Rxrα*Δex4$^{-/-}$ mice as WT (329 *vs.* 115 μM compared to 14 μM in chow fed mice; Table 1). Histologic staining showed increased necrosis in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice, but not in CA-fed WT mice (Supplementary Fig. 2). Significantly increased serum LDH levels (3.5-fold; p <0.05; Table 1) in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice compared to CA-fed WT mice confirmed this observation, altogether indicating a high BA load caused more severe liver damage in hs-*Rxrα*Δex4$^{-/-}$ mice.

Increased hepatic inflammation by CA feeding in hs-Rxrα⊿ex4$^{-/-}$ mice

CA-feeding increased *TNFα* and *IL1β* RNA 3–4-fold in hs-*Rxrα*Δex4$^{-/-}$ mice relative to the WT/chow group (Fig. 1A; p <0.05), with a similar trend for *IL6* and *F4/80*. CA-feeding induced iNOS expression 10-fold in hs-*Rxrα*Δex4$^{-/-}$ mice (Fig. 1A; p <0.05), but not in WT mice. In addition, *Ccl5* and *Tgfβ* RNA levels were 5- and 2-fold induced respectively, only in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice (Fig. 1A). Higher numbers for the pan-leukocyte marker CD45 indicated significantly increased infiltration in the CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice only (p <0.05; Fig. 1B). Taken together, livers with hepatocytes that express

Table 1. Serum liver injury markers in cholic acid-fed WT and hs-*Rxrα*Δex4$^{-/-}$ mice.

	Chow diet 5 d		1% CA diet 5 d	
Serum marker	WT	hs-*Rxrα*Δex4$^{-/-}$	WT	hs-*Rxrα*Δex4$^{-/-}$
ALT (IU/L)	13 ± 5	24 ± 3*	116 ± 56*	762 ± 605*^#
AST (IU/L)	50 ± 11	82 ± 26*	153 ± 32*	1430 ± 1626*
ALP (IU/L)	87 ± 7	71 ± 16	115 ± 14*	118 ± 19*^
LDH (IU/L)	212 ± 87	497 ± 154*	329 ± 127	1176 ± 726*^
Total bilirubin (mg/dl)	0.08 ± 0.03	0.14 ± 0.12	0.13 ± 0.07	0.62 ± 0.621
Bilirubin direct (mg/dl)	0.02 ± 0.00	0.05 ± 0.02*	0.08 ± 0.03*	0.25 ± 0.29
Indirect bilirubin (mg/dl)	0.10 ± 0.06	0.10 ± 0.08	0.07 ± 0.05	0.22 ± 0.13*
Bile acids (μmol/L)	14 ± 1	14 ± 5	116 ± 51*	329 ± 255*#

Values are expressed as average ± SD obtained from 5 to 7 animals/group.
*p <0.05 *vs.* WT/chow.
#p <0.05 *vs.* WT/CA.
^p <0.05 *vs.* KO/chow.

the RXRα-ΔDBD-variant show more inflammation and damage than those with intact RXRα.

Increased hepatic apoptosis in CA-fed hs-RxrαΔ*ex4*$^{-/-}$ *mice*

CA-fed WT mice showed increased levels of cleaved caspase 3, cleaved caspase 6 and p-JNK protein, which were elevated to a greater extent in the CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice (Fig. 2A), suggesting higher activation of apoptosis pathways in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice. Protein levels of other signaling pathways previously associated with BA-induced hepatocyte apoptosis (p-Erk, p-Akt, IkBα, 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-*Rxrα*Δex4$^{-/-}$ mice compared to CA-fed WT mice (9.6 ± 1.8 and 5.8 ± 1.8-fold *vs.* WT/chow, resp.; p <0.05; Fig. 2B), whereas Trail RNA was reduced by 50% in both genotypes (Fig. 2B). Of note, basal *DR5* expression was 2-fold (p <0.05) increased in hs-*Rxrα*Δex4$^{-/-}$ 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-*Rxrα*Δex4$^{-/-}$ mice (Supplementary Fig. 4).

Impaired BA-handling in hs-RxrαΔ*ex4*$^{-/-}$ *mice*

Many genes involved in hepatic BA adaptation are highly regulated via BA activation of FXR/RXRα-containing heterodimers. RNA levels of the direct FXR/RXRα target genes *Bsep* (*Abcb11*) and *Shp* were increased 2–3-fold (p <0.05; Fig. 3A) in CA-fed WT mice but unchanged in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice. *Ntcp* RNA was equally reduced by 80% in both CA-fed genotypes (Fig. 3A). The FXR/RXRα-regulated basolateral BA exporter complex Ostα/β showed an 18 ± 3.8-fold induction for *Ostβ* in CA-fed WT mice, with markedly impaired induction (8.2 ± 4.8-fold) in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice (Fig. 3A). *Ostα* RNA was 50% reduced in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice (Supplementary Fig. 2). The residual responsiveness of *Ostβ* expression in hs-*Rxrα*Δex4$^{-/-}$ mouse liver was further studied in primary mouse hepatocytes treated with ligands for FXR (CDCA and OCA) and RXRα (9-CisRA and LG268), and showed a 30-fold induction by CDCA and 181-fold by OCA in WT hepatocytes. Binding of FXR and RXRα to *Bsep* and *Ostβ* promoter regions followed expression patterns and was induced in CA-fed WT mice, but not in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice (Fig. 3C). A ~50% induction remained for both ligands in hs-*Rxrα*Δex4$^{-/-}$ hepatocytes (Fig. 3B). Treatment with RXRα ligands elicited minor inductions of *Ostβ* RNA in WT cells, but none in hs-*Rxrα*Δex4$^{-/-}$ hepatocytes, showing an intact RXRα-DBD is required for optimal FXR-mediated BA responsiveness for *Ostβ*.

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-*Rxrα*Δex4$^{-/-}$ *mice,* but slightly less for *Cyp8b1* (Fig. 3D). Detoxification of BA is mediated by Phase I and II enzymes. Induction of *Cyp2b10* and *Cyp3a11* in CA-fed WT mice (6.8 ± 5.7-fold and 26.8 ± 18.6-fold resp.; p <0.05; Fig. 3E) was significantly impaired in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice. *Ugt1a1* and *Sult2a1* expression was unaffected by CA-feeding WT mice but RNA levels were reduced in hs-*Rxrα*Δex4$^{-/-}$ mice (p <0.05; Fig. 3E). No changes in hepatobiliary transporter *Mrp2*, *Mrp3*, and *Mrp4* expression levels were noted by diet, genotype, or their combination (Supplementary Fig. 2).

Prolonged exposure to BA can cause fibrosis, and the markers *Col1a1* and *Timp1* were highly induced in CA-fed hs-*Rxrα*Δex4$^{-/-}$ *mice* but not in CA-fed WT mice (Fig. 3F). However, no changes in fibrosis were observed by Sirius Red analysis (Supplementary Fig. 4), likely due to the short-term duration of the study. Together these results indicate increased liver damage in hs-*Rxrα*Δex4$^{-/-}$ mice, induced by impaired BA-handling due to dysregulation of hepatic gene expression by the lack of the DBD of RXRα in hepatocytes.

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

Susceptibility to aggravated liver injury of hs-*Rxrα*Δex4$^{-/-}$ 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-*Rxrα*Δex4$^{-/-}$ mice (Supplementary Fig. 1). 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-*Rxrα*Δex4$^{-/-}$ mice (Table 2). Surprisingly, serum bilirubin induction was impaired in DDC-fed

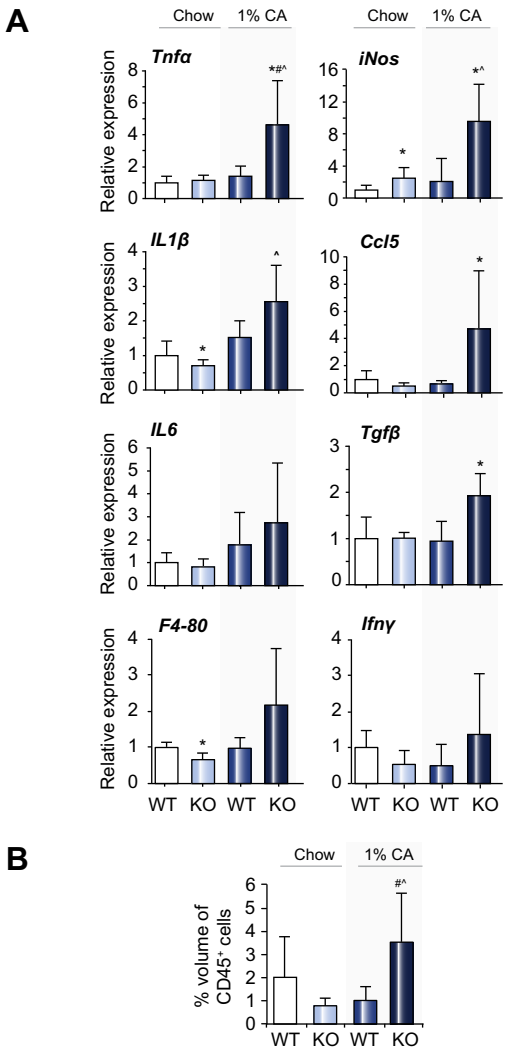

Fig. 1. Elevated hepatic inflammation in cholic acid-fed hs-*Rxrα*Δex4$^{-/-}$ mice. (A) Elevated hepatic gene expression of pro-inflammatory genes in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice as analyzed by real-time PCR. Quantification of hepatic parenchyma stained by immunohistochemistry for CD45 positive cells. (B) Values denote volume fraction (% volume) of cells of interest per hepatic parenchyma per high-power field (60× original). A total of 10 high-power fields counted per liver per mouse. Values are expressed as average ± SD obtained from 5 to 7 animals/group. $^{*}p$ <0.05 *vs.* WT/chow, $^{\#}p$ <0.05 *vs.* WT/CA, $^{\wedge}p$ <0.05 *vs.* KO/chow.

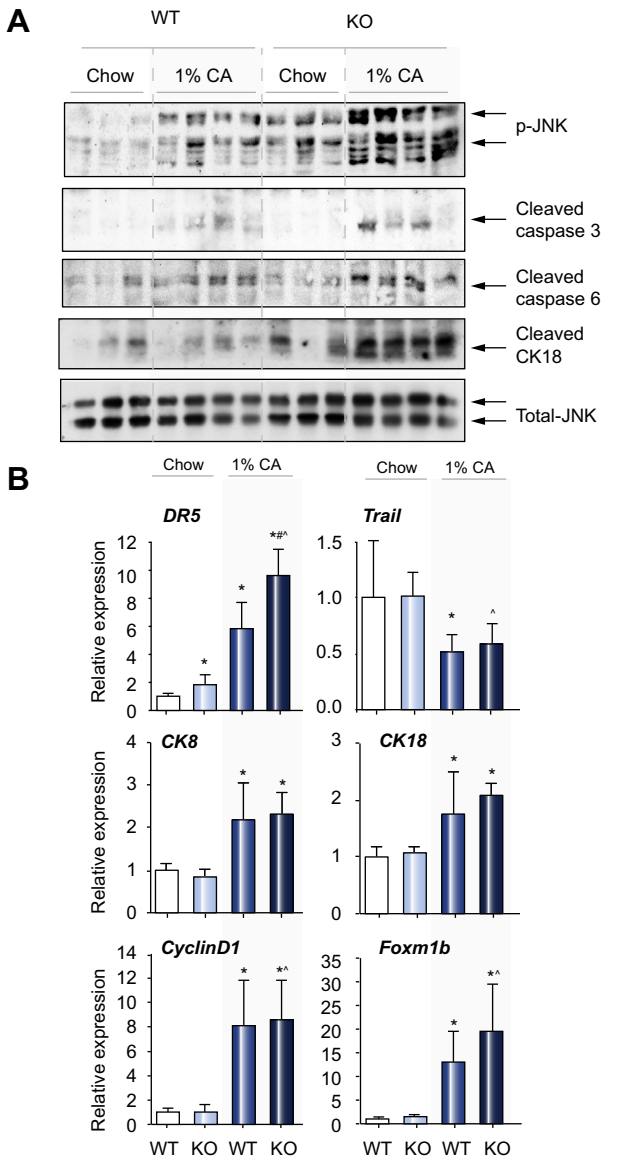

Fig. 2. Increased hepatic apoptosis in cholic acid fed hs-*Rxrα*Δex4$^{-/-}$ mice. (A) Immunoblot analyses show increased levels of p-JNK, cleaved caspases 3 and -6 and cleaved cytokeratin 18 in cytosolic fractions of livers of CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice. Total JNK serves as loading control. (B) Hepatic gene expression of pro-apoptosis markers *DR5*, *Trail*, *CK8*, *CK18* and proliferation markers *CyclinD1* and *Foxm1b* in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice. Values are expressed as average ± SD obtained from 5 to 7 animals/group. $^{*}p$ <0.05 *vs.* WT/chow, $^{\#}p$ <0.05 *vs.* WT/CA, $^{\wedge}p$ <0.05 *vs.* KO/chow.

hs-*Rxrα*Δex4$^{-/-}$ mice compared to WT mice. Despite an equal substantial elevation of serum BA (>480 μM) in both DDC-fed WT and hs-*Rxrα*Δex4$^{-/-}$ 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-*Rxrα*Δex4$^{-/-}$ mice only reached 0.67 mg/dl (Table 2). Thus, there is a discrepancy from the lack of a hepatocytic RXRα-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 *Tnfα* and *iNOS* in DDC-fed mice (Supplementary Fig. 3), apoptosis markers *DR5*, *CK8*, and *CK18* were significantly increased in DDC-fed hs-*Rxrα*Δex4$^{-/-}$ mice, indicating increased apoptosis, but not in DDC-fed WT mice (Fig. 4A). *CyclinD1* RNA showed a similar pattern, with Ki-67 staining confirming increased proliferation in WT-DDC mice but not in DDC-fed hs-*Rxrα*Δex4$^{-/-}$ mice (Supplementary Fig. 4). No changes in p-JNK, cleaved caspase 3 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-*Rxrα*Δex4$^{-/-}$ mice. The induction of *Ostβ* in DDC-fed WT mice was impaired in hs-*Rxrα*Δex4$^{-/-}$ mice *Cyp7a1* RNA was markedly reduced in DDC-fed WT mice, with a more modest reduction in hs-*Rxrα*Δex4$^{-/-}$ mice (Fig. 4B). ChIP-QPCR analysis for FXR and RXR binding on the *Bsep* and *Ostβ* promoters did not fully reflect the changes in *Bsep* RNA expression (Fig. 4C). These results may

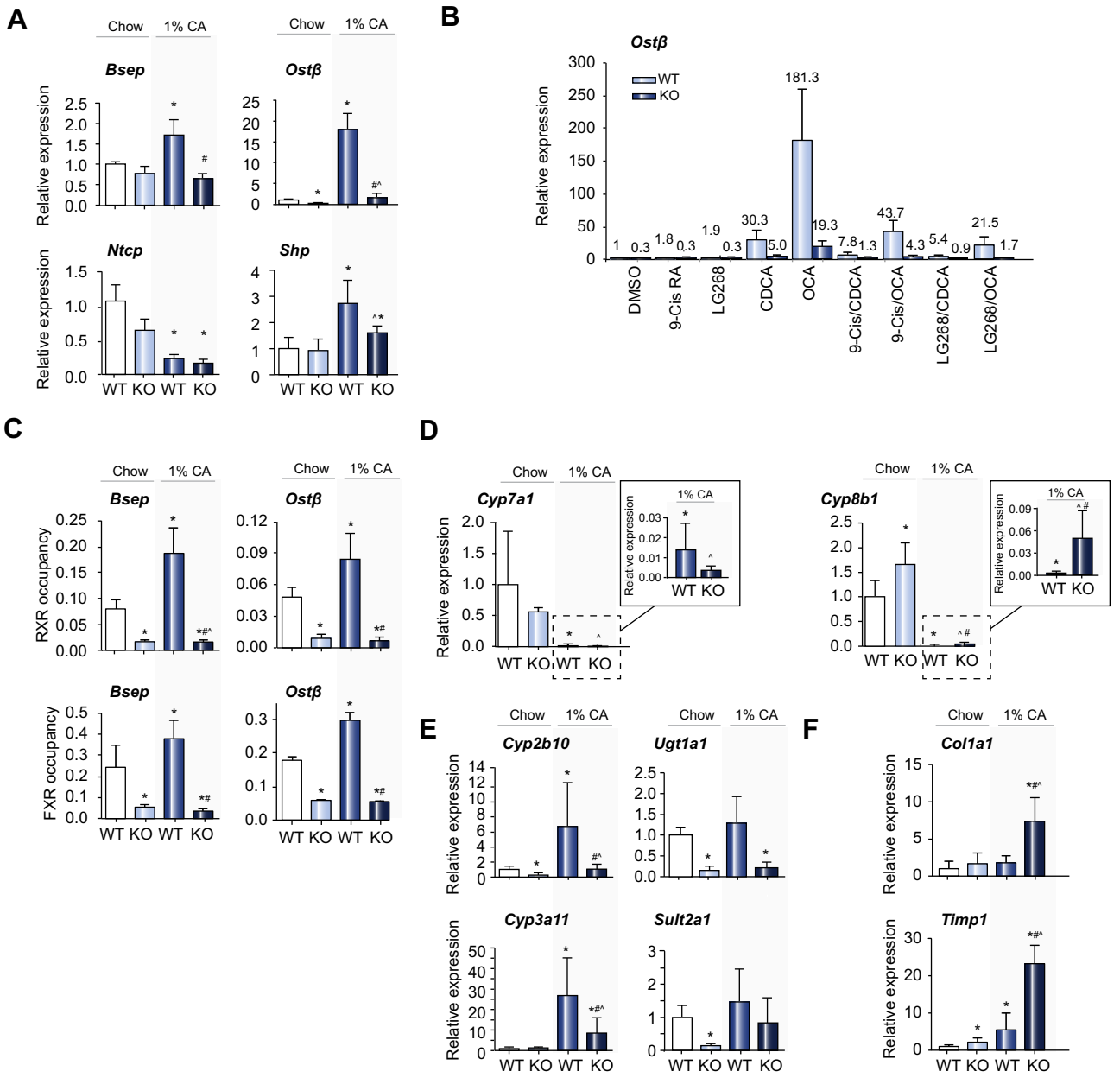

Fig. 3. Impaired regulation of gene expression involved in hepatic BA handling in cholic acid-fed hs-*Rxr*αΔex4$^{-/-}$ mice. (A) Impaired regulation of genes involved in hepatic BA transport in CA-fed hs-*Rxr*αΔex4$^{-/-}$ mice (B) or in primary hepatocytes. (C) Hepatic FXR and RXRα binding to *Bsep* and *Ostβ* promoter regions in CA-fed hs-*Rxr*αΔex4$^{-/-}$ mice. (D) Impaired regulation of genes involved in hepatic BA synthesis and (E) BA detoxification in CA-fed hs-*Rxr*αΔex4$^{-/-}$ mice (F). Values are expressed as average ± SD obtained from 5 to 7 animals/group. $^{*}p$ <0.05 *vs.* WT/chow, $^{\#}p$ <0.05 *vs.* WT/CA, $^{\wedge}p$ <0.05 *vs.* KO/chow.

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 *Col1a1* and *Timp1* by DDC in both genotypes (Fig. 4D), fibrosis was reduced in DDC-fed hs-*Rxr*αΔex4$^{-/-}$ mice (Supplementary Fig. 4). Taken together, there were many commonalities in the roles for the RXRα-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 RXRα.

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 RXRα-ΔDBD under inflammatory conditions, likely caused by activity through differential interactions of the internally truncated protein with its partners. This prompted us to delineate the residual ability of RXRα-partners to be activated, with a

Table 2. Serum liver injury markers in DDC-fed WT and hs-*Rxrα*Δex4$^{-/-}$ mice.

	Chow diet 3 weeks		0.1% DDC diet 3 weeks	
Serum marker	WT	hs-*Rxrα*Δex4$^{-/-}$	WT	hs-*Rxrα*Δex4$^{-/-}$
ALT (IU/L)	31 ± 14	73 ± 51	1321 ± 826*	1044 ± 463*^
AST (IU/L)	84 ± 47	109.13 ± 50.28	1285.44 ± 788.12*	965.09 ± 399*^
ALP (IU/L)	109 ± 9	135.38 ± 106.71	335 ± 83.41*	400 ± 157*^
LDH (IU/L)	319 ± 149	328.25 ± 156.65	1608.11 ± 923.08*	1403.64 ± 585.07*^
Total bilirubin (mg/dl)	0.15 ± 0.032	0.3 ± 0.11	4.94 ± 2.11*	0.67 ± 0.25^#
Bilirubin direct (mg/dl)	0.04 ± 0.0098	0.1 ± 0.047*	3.51 ± 1.50*	0.21 ± 0.087#
Indirect bilirubin (mg/dl)	0.1 ± 1.50 E-17	0.22 ± 0.10*	1.42 ± 0.64	0.45 ± 0.16
Bile acids (μmol/L)	14 ± 2	37 ± 23	498 ± 693*	486 ± 287*^

Values are expressed as average ± SD obtained from 5 to 7 animals/group.
*p <0.05 *vs.* WT/chow.
#p <0.05 *vs.* WT/DDC.
^p <0.05 *vs.* KO/chow.

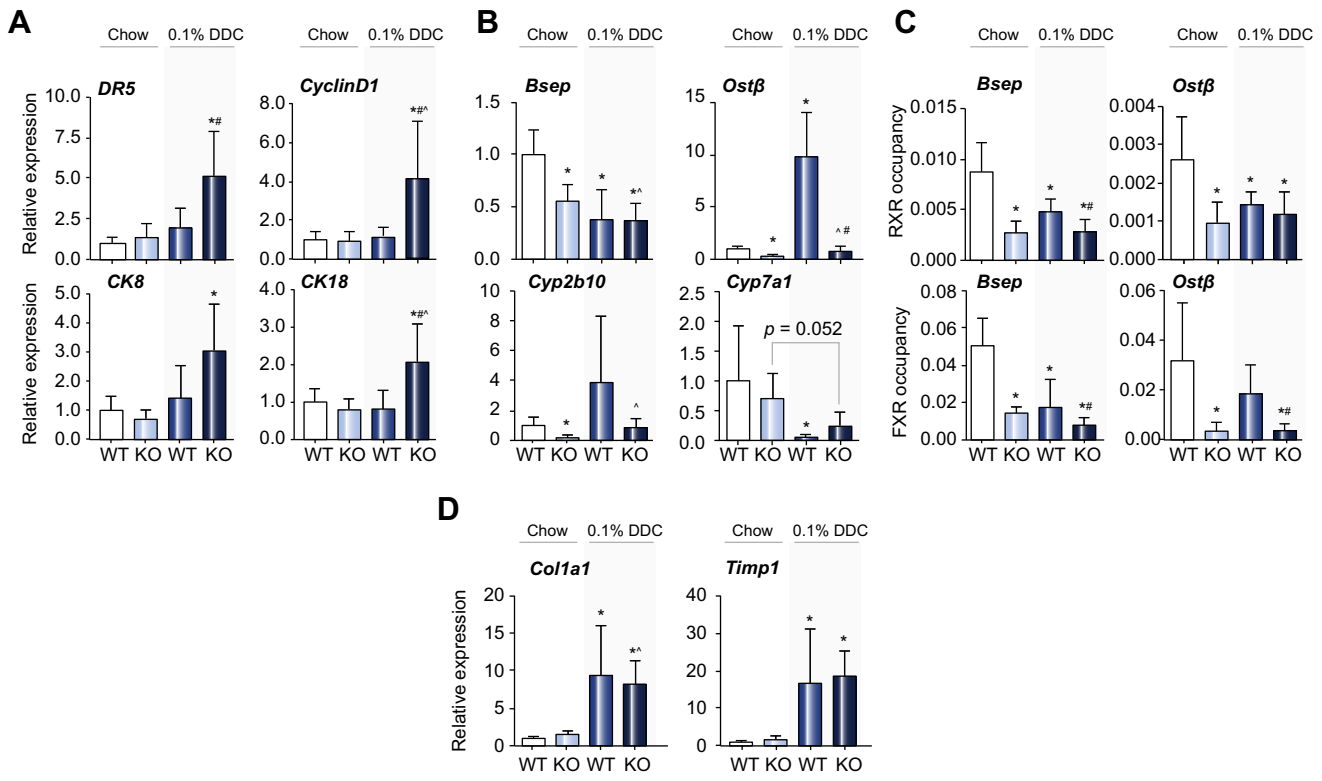

Fig. 4. Gene expression involved in hepatic apoptosis and bile acid handling in DDC-fed hs-*Rxrα*Δex4$^{-/-}$ mice. (A) Hepatic gene expression of pro-apoptosis markers *DR5*, *CK8*, *CK18* and proliferation marker *CyclinD1*, in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice. (B) Impaired hepatic gene expression of NR-regulated genes involved in hepatic BA transport and metabolism in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice. (C) Hepatic FXR and RXRα binding to *Bsep* and *Ostβ* promoter regions in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice and (D) fibrosis markers in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice (D). Values are expressed as average ± SD obtained from 5 to 7 animals/group. *p <0.05 *vs.* WT/chow, #p <0.05 *vs.* WT/CA, ^p <0.05 *vs.* KO/chow.

specific focus on NR-activation by BA and the subsequent physiological adaptation in this study.

Overall, liver injury in both models was accompanied by markedly elevated serum biochemistry markers (ALT, AST, bilirubin), increased serum BA levels, elevated pro-inflammatory, profibrotic and pro-apoptotic responses, along with evidence of increased necrosis (histology and LDH), and finally an impaired hepatic adaptive gene expression response, showing that RXRα-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-*Rxrα*Δex4$^{-/-}$ mice. This finding is intriguing, and to date not fully explained by the adaptive response of 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 RXRα.

Induction of *Ostβ* and *Cyp2b10* in hs-*Rxrα*Δex4$^{-/-}$ mice was impaired in both CA and DDC cholestatic models, suggesting similar adaptive responses. While basal *Ostβ* and *Cyp2b10* RNA levels were 70% lower in chow-fed hs-*Rxrα*Δex4$^{-/-}$ mice, confirming

previous studies [8], RNA levels could be partially induced by CA and DDC-feeding in hs-*Rxrα*Δex4$^{-/-}$ mice. This residual induction was confirmed in primary mouse hepatocytes treated with FXR ligands, but no induction by RXRα ligands was noted, indicating the remaining functionality of the RXRα-ΔDBD 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 contribution to the observed aggravated hepatic injury in CA-fed hs-*Rxrα*Δex4$^{-/-}$ mice is the enhanced pro-inflammatory response (*TNFα*, *iNOS*, *IL1β*, *Ccl5*). Whether this is due to direct or indirect roles for RXRα and/or BA needs further study. The reported anti-inflammatory actions of BA through activation of FXR [18], as partner of RXRα, 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-*Rxrα*Δex4$^{-/-}$ 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-*Rxrα*Δex4$^{-/-}$ 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-*Rxrα*Δex4$^{-/-}$ 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-*Rxrα*Δex4$^{-/-}$ 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 RXRα and precursors for its ligand (vitamin A and 9-Cis RA respectively) in fibrosis though with conflicting results. Some studies have shown that 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 PPARδ, LXRα/β, VDR [23–26], are involved in fibrosis. Interestingly however, this was shown in stellate cells, whereas in our model RXRα 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 RXRα affects regulation of these genes remains to be determined.

Since RXRα is the obligate partner for several NRs functioning as BA-activated receptors (FXR, CAR, PXR, and VDR) it could very well be considered that RXRα 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-*Rxrα*Δex4$^{-/-}$ [29–32]. It should also be considered that these were whole mouse knockout models, whereas in our study the DBD of RXRα was specifically deleted in hepatocytes, and may therefore serve as a more specific model for studying roles played by fully-functional RXRα heterodimers.

Previous studies treating hs-*Rxrα*Δex4$^{-/-}$ mice with the ligand for another partner of RXRα, PPARα, 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-*Rxrα*Δex4$^{-/-}$ mice [34]. Differential responses of *Cyp3a11*, *CyclinD1*, and *IL1β* RNA between those studies and ours indicate the role of DNA binding of RXRα 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 RXRα-DBD.

In conclusion, we show aggravated liver injury in hs-*Rxrα*Δex4$^{-/-}$ mice after high BA load, indicating the DBD of RXRα 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-*Rxrα*Δex4$^{-/-}$ 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-*Rxrα*Δex4$^{-/-}$ mice. Hepatocyte RXRα 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 RXRα heterodimers in the hepatic adaptive response to cholestasis.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2013.09.026.

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