Published in final edited form as:

Liver Int. 2012 February ; 32(2): 321–330. doi:10.1111/j.1478-3231.2011.02664.x.

Gene-specific alterations of hepatic nuclear receptor regulated gene expression by ligand activation or hepatocyte-selective knockout inhibition of RXR α signaling during inflammation

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Abstract

Background—Inflammation leads to transcriptional downregulation of many hepatic genes, particularly those activated by RXR α -heterodimers. Inflammation-mediated reduction of nuclear RXR α levels is a main factor in reduced nuclear receptor (NR)-regulated hepatic gene expression, eventually leading to cholestasis and liver damage.

Aim—To investigate roles for RXR α in hepatic gene expression during inflammation, using two complementary mouse models: ligand-activation of RXR α , and in mice expressing hepatocyte-specific expression of RXR α missing its DNA-binding-domain (DBD; *hs-Rxr ex4*−/−)

Methods—To activate RXR α , mice were gavage-fed with LG268 or vehicle for 5 days. To inhibit RXR α function, *hs-Rxr ex4*−/− were used. All mice were IP-injected with LPS or saline for 16 hrs prior to analysis of hepatic RNA, protein and NR-DNA binding.

Results—LG268-treatment attenuated the LPS-mediated reductions of several RXR α -regulated genes, coinciding with maintained RXR α occupancy in both *Bsep* and *Ost* promoters. Lacking full hepatocyte-RXR α function (*hs-Rxr ex4*−/− mice) led to enhancement of LPS-mediated changes in gene expression, but surprisingly, maintenance of RNA levels of some RXR α -regulated genes. Investigations revealed that *Hs-Rxr* −/− hepatocytes expressed an internally-truncated, ~44 kDa, RXR α -form. DNA-binding capacity of NR-heterodimers was equivalent in wt and *hs-Rxr ex4*−/− livers, but reduced by LPS in both. ChIP-QPCR revealed reduced RXR α occupancy to the *Bsep* RXR α :FXR site was reduced, but not absent, in *hs-Rxr ex4*−/− livers.

Conclusions—There are differential regulatory roles for hepatic RXR α , both in basal and inflammatory states, suggesting new and complex multi-domain roles for RXR α in regulating hepatic gene expression. Moreover, there is an unexpected non-obligate role for the DBD of RXR α .

Keywords

liver; inflammation; nuclear receptor; RXR α ; mouse; LG268; rexinoid; gene expression; DNA-binding

Introduction

Inflammation induced-cholestasis is associated with hepatic and systemic accumulation of toxic biliary compounds including bile acids, xenobiotics and bilirubin which, if prolonged, leads to jaundice and liver damage (1–3). Lipopolysaccharide (LPS) released by gram-negative bacteria is one of the primary inducers of the physiologic manifestations of sepsis and induces a broad range of changes in hepatic gene expression, including the acute phase response (APR), of which cholestasis is a central component (4–5). In hepatocytes, the negative APR involves downregulation of the main metabolic pathways, energy generation, drug metabolism and detoxification as well as the expression of transporter proteins regulating uptake and secretion of most biliary components. The majority of these genes are transcriptionally regulated by type 2 nuclear receptors (NRs), which are ligand-activated transcription factors that require heterodimerization with Retinoid X receptor (RXR ; NR2B1) for full activity. Studies from our laboratory as well from others (6–15) have demonstrated inflammatory signals such as LPS and cytokines (primarily TNF and IL-1) reduced hepatic nuclear RXR protein levels in vivo and in vitro. By virtue of being a common heterodimeric partner of multiple NRs, reduced quantities and functionality of nuclear RXR appears to be a major contributor of impaired hepatic gene expression during negative hepatic APR. On the other hand, activation of many of the partners of RXRs is integral to hepatoprotection in cholestasis and toxin-related injuries. However roles for, and relevance of RXR function in inflamed liver and whether there is opportunity for modulation of RXR function with rexinoids, have not been not completely clarified. This study investigated a further role for RXR in regulation of hepatic gene expression under inflammatory conditions in two complementary mouse models: one, where RXR selectively is impaired in hepatocytes by deletion of the DNA-binding domain (DBD) of RXR , and a second model where RXR was activated with the synthetic ligand (rexinoid) LG268.

Methods

Animal experiments

Mice were maintained in a temperature- and humidity-controlled environment and provided with water and rodent chow ad lib. Eight week old male mice were used for all experiments. All animal protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Hepatocyte-specific-*Rxr exon4*^{-/-} (*hs-Rxr exon4*^{-/-}) mice (16) or wild-type (wt) mice on a mixed C57Bl/6-DBA2-129SV background, were created by deletion of exon4 which encodes the DNA-binding domain of RXR . Male *hs-Rxr exon4*^{-/-} and wt mice were injected intraperitoneally with LPS (*Salmonella typhimurium*; Sigma Chemical Co., St. Louis, MO) at dose of 2 mg/kg or equivolume administration of 0.9% saline. Livers were harvested 1 or 16 hrs later. Male C57/BL6 mice were gavage-fed LG268 (30/mg/kg/day) or vehicle (CMC/Tween-80/PEG400) for 5 days (17) followed by LPS or saline administration as above. Livers were harvested 1 or 16 hrs later. For all liver tissues RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's protocol, and cDNA was made using High throughput Kit (Applied Biosystems, Applied Biosystems, Inc., Foster City, CA). Gene expression was determined as described before. Primers and probes were obtained from Sigma Genosys and sequences are listed as supplemental data. All data were analyzed by Two-Way ANOVA. p-values < 0.05 were considered significant.

Protein analysis

Nuclear fractions were obtained as previously described and western blot analysis for RXR was performed as before (18).

EMSA

Nuclear fractions were isolated and EMSAs were performed as described previously (7)

Biotin-IP assays were based on Xu et al (19). 40 µg of liver nuclear extracts were incubated with 1 µg of biotinylated promoter-specific ds oligonucleotide, 10 µg poly dI-dC and 5x Binding buffer (125 mM Hepes, pH 7.6, 250 mM KCl, 25 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 50% glycerol) and incubated overnight at 4 °C with rotation. Protein bound to biotinylated oligos were pulled down by incubation of 25 µl streptavidin-agarose beads (Pierce) for 1–2 hrs at 4 °C with rotation and beads spun down for 2 min at 8000g, 4 °C, washed 4x with 100 µl cold HKMG buffer (10 mM HEPES pH7.9, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 1mM DTT, 0.5% NP-40) containing phosphatase inhibitors and protease inhibitors. Laemmli sample buffer was added to elute bound protein and subjected to immunoblot analysis using RXR and FXR antibodies.

Chromatin Immunoprecipitation (ChIP)

Frozen mouse liver was grinded in fine powder and was crosslinked in 1% formaldehyde and quenched with 125 mM glycine. The tissue was then homogenized in nuclear isolation buffer and pellet was resuspended in nuclear lysis buffer. Chromatin concentrations were adjusted to 2 µg/ul. Chromatin was sheared to 200–500 bp (Bioruptor 300 (Diagenode, Sparta, NJ). 30 µg chromatin was precleared with protein-A agarose beads. The supernatant was subjected to immunoprecipitation, an 30 ul aliquot was taken as input for later use. Four µg RXR or FXR polyclonal antibody (Santa Cruz) was added to the chromatin lysate and incubated overnight at 4°C. As a negative control, nonimmune rabbit IgG was used in place of specific antibodies. Immune complexes were precipitated by the addition of protein-A agarose beads at 4°C. The beads were washed in the order of low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and TE buffer. Immune complexes were finally eluted in a 1% SDS/100 mM NaHCO₃ buffer at room temperature. The final elute was treated by 0.2M NaCl, 0.05µg/µl RNase for 1 hour, followed by 0.1 µg/µl Proteinase K overnight at 65°C to reverse the DNA- protein crosslinks. The resulting DNA and input DNA was purified using PCR purification columns (Qiagen, Valencia, CA). Levels of transcription factors occupancy at differential target gene promoters were determined by SYBR Green Realtime PCR (Applied Biosystem, Carlsbad, CA). Input and immunoprecipitated DNA reactions were performed in duplicate. The resulting signals relative to Input DNA were expressed as mean ± SD.

Results

Canalicular transporter gene expression

Multiple studies have established that inflammatory signals alter gene expression levels of canalicular hepatobiliary transporters (5). To determine roles for inhibition and activation of RXR in the regulation of these genes, their RNA expression levels were examined in mice given the rexinoid LG268 as well as in hepatocyte-specific *Rxr* knockout mice (*hs-Rxr ex4-/-*) under inflammatory conditions. In wild-type (wt) mice and in vehicle-pretreated mice, RNA expression levels of canalicular transporters *Bsep*, *Abcg5*, *Abcg8*, *Mdr2*, *Mrp2*, were 54–80% reduced ($p < 0.05$), 16 hours after LPS treatment (Figure 1 and S1). RNA levels of *Bsep* and the *Abcg5/g8* heterodimer were similarly reduced in *hs-Rxr ex4-/-* mice given LPS, whereas LPS further reduced *Mdr2* expression in *hs-*

Rxr *ex4*^{-/-} mice to 25% of wt mice ($p < 0.05$). *Mdr1b* RNA expression was upregulated 2-fold by LPS in wt mice, which was significantly enhanced in LPS-treated *hs-Rxr* *ex4*^{-/-} mice to 2.85 fold ($p < 0.05$; Figure 1).

A trend towards attenuation by LG268 pretreatment was observed on the LPS-mediated downregulation of *Bsep* (Figure 1). The downregulation by LPS of *Abcg5*, *Abcg8* and of *Mdr2*, but not *Mrp2*, was completely prevented by rexinoid activation of RXR prior to LPS treatment (Figure 1). No effects on basal expression were observed by LG268-activation of RXR or by impaired RXR function, with the exception of a 50% reduction in *Mdr2* in *hs-Rxr* *ex4*^{-/-} mice ($p < 0.05$). *Fic1* RNA expression was not affected by LPS nor inhibition or activation of RXR (Figure S1).

Sinusoidal transporters

The bile acid uptake transporters *Ntcp*, *Slco1a1* and *Slco1a4* were significantly downregulated by LPS (50–85%, $p < 0.05$) in both wild-type and vehicle pretreated mice (Figure 2 and S1). A similar downregulation in *hs-Rxr* *ex4*^{-/-} or LG268 pretreated mice was observed compared to wt mice for *Ntcp* and *Slco1a4* RNA levels (Figure S1). A slight (10%) but significant attenuation in LPS-mediated reduction was observed for *Slco1a1* expression in livers of *hs-Rxr* *ex4*^{-/-} mice (Figure 2). Basal levels of *Slco1a4* were 2-fold higher ($p < 0.05$) in *hs-Rxr* *ex4*^{-/-} mice (Figure 2). In addition to a 73% reduction in baseline levels of *Slco1a1* RNA ($p < 0.05$) by LG268 alone, the pretreatment further reduced LPS-mediated repression of *Slco1a1* RNA expression to 90% compared to vehicle (80% downregulation, Figure 2). The expression of the basolateral bile acid efflux transporters *Mrp3* and *Mrp4* RNA levels were reduced by LPS by 50–70% ($p < 0.05$) (Figure 2). In addition, *Mrp3* RNA levels were 62% lower in livers of *hs-Rxr* *ex4*^{-/-} mice compared to wild-type mice and were further reduced to 90% by LPS in *hs-Rxr* *ex4*^{-/-} mice ($p < 0.05$). *Mrp4* RNA expression was similarly reduced by LPS in *hs-Rxr* *ex4*^{-/-} mice compared to wild-type mice. Activation of RXR by LG268 did not affect *Mrp3* expression, or the reduced expression by LPS (Figure 2). In contrast, *Mrp4* levels were 2-fold induced by LG268 ($p < 0.05$), but no interference of LG268 on the LPS-mediated *Mrp4* RNA level reduction was noted (Figure 2). Interestingly, the expression of the heterodimeric transporters genes *Ost* and *Ost* were not significantly affected by LPS. *Ost* RNA expression was not affected by LG268-treatment or genotype (Figure S1). Baseline expression of *Ost* RNA was 80% reduced ($p < 0.05$) in absence of RXR, and this was not affected by LPS treatment. In contrast LPS treatment in combination with RXR-activation by LG268 pretreatment resulted in a 6-fold induction of *Ost* RNA expression (Figure 2) enhancing the 2-fold induction ($p < 0.05$) of LG268 pretreatment alone.

Bile acid synthesis

The LPS-induced downregulation of *Cyp8b1* was comparable in wt mice and *hs-Rxr* *ex4*^{-/-} mice (80%, Figure 3). In contrast, RXR activation with LG268 further reduced LPS-mediated *Cyp8b1* RNA levels by an additional 10% ($p < 0.05$). Neither absence of RXR or activation of RXR interfered with the 70–90% LPS-induced downregulation of *Cyp7a1* RNA (Figure S1).

Phase I detoxification

LPS did not significantly affect *Cyp2b10* RNA expression in wt mice or vehicle pretreated mice (Figure 3), whereas *Cyp3a11* RNA levels were reduced by 70%. *Cyp2b10* basal RNA expression was regulated in opposite manners by activation and inhibition of RXR function and showed an 80% reduction ($p < 0.05$) in livers of *hs-Rxr* *ex4*^{-/-} mice, which was attenuated in by LPS treatment, and a 3.6-fold upregulation by LG268 (Figure 3). LPS treatment did not further modify this induction. Basal expression of *Cyp3a11* RNA was 2.3-

fold ($p < 0.05$) induced in *hs-Rxr ex4-/-* mice and the LPS-mediated reduction was attenuated to an 80% reduction. Pretreatment of LG268 prior to LPS-treatment showed a similar downregulation of *Cyp3a11* RNA levels as vehicle pretreatment and did not affect basal levels of *Cyp3a11* gene expression.

Phase II detoxification

Sult2a1 RNA expression showed no significant changes by LPS in both wt mice and vehicle pretreated mice (Figure 3). In wt mice LPS reduced *Ugt1a1* RNA expression to 40% of saline treatment ($p < 0.05$), which was further reduced to 13% ($p < 0.05$) when *hs-Rxr ex4-/-* mice were given LPS. Basal expression of *Sult2a1* and *Ugt1a1* were reduced by 80 and 70% respectively in *hs-Rxr ex4-/-* mice, which was further suppressed in the *hs-Rxr ex4-/-* mice. LG268 pretreatment induced *Sult2a1* RNA expression 6-fold ($p < 0.05$), which when treated with LPS, was not significantly different from LG268 alone. LG268 pretreatment attenuated the reduction of *Ugt1a1* by LPS (Figure 3).

Regulation of RXR- dependent gene expression and nuclear extract binding capacities of RXR α -containing NR heterodimers

These results show differential responses in gene expression with respect to the involvement of RXR and engagement of rexinoid-sensitive pathways. EMSAs performed using the consensus NR-binding sites DR-1 to DR-5 (data not shown), and IR-1 (Figure 4A) and promoter-specific IR-1 sites of Bsep, Ost and Ost genes (data not shown) for both experimental models showed reduced binding of nuclear fractions after 16 hours of LPS treatment (Figure 4A), consistent with previously published results (7). No significant interfering effects of activation by rexinoids or inhibition with DBD-selective excision of RXR on reduced binding in liver nuclear extracts by LPS were seen. No difference in nuclear binding capacity between vehicle and LG268 treatments was observed either. Surprisingly, no noteworthy difference in binding between wt and *hs-Rxr ex4-/-* mice was observed, which is inconsistent with expected results since deletion of the DBD of RXR would have been expected to render any remaining RXR protein unable to bind DNA and thus be non-functional.

Nuclear RXR protein levels were investigated by Western blot. Previously we and others have shown that hepatic nuclear RXR levels are reduced and modified by LPS treatment, with the most profound effects after 1 hr of LPS treatment (7, 18). In wt mice, 1 hour after administration of LPS, the expected 55 kDa RXR showed a similar reduction and modification (Figure 4B)(7, 18), whereas a band of this size was absent in the *hs-Rxr ex4-/-* mice. However, a lower molecular weight band of ~ 44 kDa appeared specifically in the *hs-Rxr ex4-/-* liver nuclear extracts (Figure 4B). In silico examination of the construct used to create the mice with deletion of RXR -exon 4, indicated an in-frame deletion, resulting in a protein with a predicted size of 44 kDa. This indicated the possibility of the presence of an RXR protein with an internal deletion of exon 4, thus missing the RXR DBD, while other domains remain intact. Quantification of RNA levels of RXR in wt and *hs-Rxr ex4-/-* mice by QPCR using primers located in exon 6 and 7, after the deleted exon4, showed equal RNA levels (Figure S3). In addition, using an additional antibody for RXR directed against the C-terminal part of RXR showed the same size band (Figure S3), confirming this to be an internally truncated RXR protein, without a DBD. As can be seen from Figure 4B, the mutated RXR -exon4 protein responded in a similar fashion to short-term LPS treatment as the wild type RXR, with reduction and modification. Pretreatment of wt mice with LG268 reduced RXR levels and also caused RXR to modify, to a lesser extent compared to LPS, and did not interfere with the LPS-mediated changes (Figure 4B).

To determine if the internally-truncated RXR α protein is able to interact with partners and still able to participate in the heterodimeric DNA-binding complex and stimulate gene expression, biotin-pulldown assays followed by western blot were performed on *Bsep*-IR and *Ost*-IR-1 specific sequences (12, 20). DNA binding was observed for both RXR α wt and RXR α DBD mutant to these sequences in vitro, indicating capability of occupancy of the site (either direct or indirect) by the mutant RXR α protein. In addition FXR binding could be detected, which was not different between wt and *hs-Rxr ex4*^{-/-} mice suggesting the likelihood of binding of RXR α to DNA through its interactions with FXR via other domains. ChIP assays for RXR α and FXR binding on the same IR-1 site in mouse *Bsep* promoter showed a 60% reduction ($p < 0.05$) in binding of RXR α in wt mice after 1 hr of LPS treatment with a similar change for FXR binding under the same conditions. Binding of both FXR and RXR α was reduced by 70% and 80% resp ($p < 0.05$) in *hs-Rxr ex4*^{-/-} mice compared to wt mice. FXR and RXR α binding after LPS treatment was further reduced vs saline *hs-Rxr ex4*^{-/-} mice. RXR α but not FXR binding to the *Ost* promoter was reduced by 50% ($p < 0.05$) by LPS treatment in wt mice. However, a 90% reduction ($p < 0.05$) in binding for both RXR α and FXR was observed in *hs-Rxr ex4*^{-/-} mice compared to wt mice. LPS treatment of *hs-Rxr ex4*^{-/-} mice did not result in additional changes in binding of FXR and RXR α to this site. Thus, although both *Bsep* and *Ost* have strong IR-1 elements bound by RXR α :FXR heterodimers, they respond differently when the DBD of RXR α is deleted.

RXR α activation by LG268 enhanced binding of RXR α to the *Bsep* promoter by 2.5 fold ($p < 0.05$) and it remained activated in LG268/LPS treated mice. The reduced binding of FXR on the *Bsep* promoter by LPS was also prevented in mice pretreated with LG268. RXR α binding to the *Ost* promoter was 1.5-fold induced ($p < 0.05$) by LG268 and LPS treatment showed a tendency to enhanced binding over LG268 alone. Binding of FXR to the *Ost* promoter site was not altered by LPS or LG268.

Discussion

Many hepatic functions, including sinusoidal bile acid uptake and excretion, intracellular metabolism and canalicular secretion, are controlled by genes of which the expression is primarily governed by nuclear receptors that function through heterodimerization with RXR α . RXR α as such, serves as a master regulator for many physiological processes in liver and any alterations in RXR α will lead to changes in hepatic function. Inflammation-induced changes in RXR α function alter hepatic expression of transporters and bile acid detoxification genes resulting in cholestasis and liver damage, supporting the need to not only fully understand the underlying mechanisms, but also to determine the role for of RXR α within each heterodimerization complex and responses to inflammation (explored via the hepatic-specific *Rxr ex4*^{-/-} mouse), as well as intervention with RXR α ligands. By exploring these effects in complementary mouse models, the results of these studies indicate that activation and inhibition of RXR α function in liver results in disparate effects: lack of full RXR α function in hepatocytes aggravated the LPS-mediated changes in gene expression, while activation of RXR α by the rexinoid LG268 attenuated these effects. However, there was an unexpected complexity to this paradigm, where there were gene-specific, and not global, effects on RNA levels of RXR α -dependent genes in the DBD-deficient (*hs-Rxr ex4*^{-/-}) mice, response to LPS, and after treatment with the rexinoid LG268. These results provide new windows into the roles and functioning of RXR α and its domains that interact and function with partners, with specific new insights into the non-obligate role for the DBD of RXR α to support full functionality.

Moreover, these results suggest RXR α activation can, at least partially help maintain bile formation under inflammatory conditions, specifically cholesterol and phosphatidylcholine

secretion, since LG268 nearly completely prevented the LPS-induced downregulation of *Abcg5*, *Abcg8* and of *Mdr2*, with a slight attenuation on downregulation of *Bsep* RNA expression. In addition, RXR activation by LG268 could result in an additional reduction of intracellular BA levels and potentially attenuating liver damage with enhanced downregulation of BA uptake at the sinusoidal membrane via *Slco1a1* gene expression, in combination with a significantly 6-fold enhanced upregulation of *Ost* RNA under inflammatory conditions. RXR activation by LG268 improved overall liver detoxification during inflammation, as indicated by enhanced reduction *Cyp8b1*, increased *Cyp2b10* and *Sult2a1* by LG268 when cotreated with LPS, and attenuated downregulation of *Ugt1a1*. In contrast, in mice with hepatocyte-specific deletion of RXR-DBD appear to get a more aggravated liver gene expression profile when challenged with LPS. Sinusoidal efflux of BA through *Mrp3* and *Ost* appeared impaired since the expression of both genes was reduced at both basal levels and after LPS treatment in *hs-Rxr ex4-/-* mice. The minor attenuation of LPS-mediated downregulation of *Slco1a1* RNA in *hs-Rxr ex4-/-* mice, suggests that BA uptake may be increased. Mixed results were observed on detoxification genes and bile flow: on the one hand BA hydroxylation function appears improved as indicated by the upregulation of *Cyp3a11* RNA in *hs-Rxr ex4-/-* mice administered LPS. On the other hand *Ugt1a1* and *Sult2a1* RNA expression levels were reduced in these mice, implying reduced glucuronidation and sulfation of toxic compounds that are accumulating under these inflammatory conditions in absence of RXR, correlating with the observation that serum bilirubin levels are elevated in *hs-Rxr ex4-/-* mice, however this not affected by LPS treatment. Slight aggravation in the reduction in *Mdr2* RNA expression, but enhanced the induction of *Mdr1b* RNA compared to wt mice.

With RXR being the common heterodimeric partner for at least a dozen NRs in liver, many NR-target genes were expected to change. Unexpectedly, we observed only a small number of alterations in gene expression in this study with either RXR activation or with reduced function of RXR, and interestingly those changes were not restricted to genes activated by one specific partner of RXR. Therefore instead of a correlation between changes in gene expression and permissive and non-permissive partners of RXR, these data suggest a gene-specific function for RXR with a more complex mechanism of regulation of gene expression, and separate roles for RXR domains. Several recent studies confirm these concepts of multiple contributions to RXR-containing heterodimers functions (21). Detailed structural information is available for isolated DBDs and Ligand Binding Domains (LBDs) of nuclear receptors, including RXR (22–28), but the full crystal structure of only the PPAR/RXR heterodimers is available and supports multiple points of contact between partners, not just via the LBD (29). In addition, information on interaction of NR-heterodimers with coregulators is derived from studies using peptides of the domain containing the LXXLL motif, which interacts with the nuclear receptor proteins (30–31). Our data may suggest the remaining domains of the internally mutated RXR exon4 protein can heterodimerize, and likely recruit coregulators, albeit at an attenuated rate for individual sites (30–31). This likely provides an explanation for the modest effects on gene expression for many genes, well as for lack of difference in binding of nuclear fractions on EMSA between wt and *hs-Rxr ex4-/-* mice. Since the DBD is deleted in-frame, but the LBD heterodimerization domains are intact, interaction with its partners is somewhat preserved. Our data indeed indicated that deletion of the DBD retained capacity to bind to the DNA either directly or, more likely as a component of the complex interactions with partner (which still has its DBD) or coregulators (as shown by EMSA and Biotin-pull down assays). Thus, these data indicate that the DBD of RXR may not be essentially required for activation of gene transcription for at least a subset of genes. Alternative explanations such as compensation by RXR or RXR seems unlikely since no changes of RNA(16) nor protein levels were observed (Kosters and Karpen unpublished) in the *hs-Rxr ex4-/-* mice. Since RXR is mutated only in hepatocytes, but remains intact in other liver cell types

such as Kupffer cells and stellate cells, this could explain why binding would be changed all that much. However our Western blot data in Fig 4 shows that a 55kD band is absent, making this second alternative explanation very unlikely. Further studies (e.g X-ray crystallography, coregulator ChIP studies, other partners) are clearly needed to explore and clarify roles for the non-DBD domains of RXR in heterodimerization, binding site occupancy, and function.

The reduced occupancy of both RXR and FXR 1 hour after LPS treatment on the *Bsep* promoter coincided with reduced gene expression (~ - 80%) after 16 hrs LPS in wt mice. In contrast, a small, but insignificant change in *Bsep* gene expression was seen in *hs-Rxr ex4-/-* mouse livers (~-20%), but both FXR and RXR DNA occupancy was substantially reduced (by ~ -60 to 70%), suggesting factors in addition to full maintenance of RXR:FXR occupancy, or the presence of accommodation to reduced binding, are involved in basal transcription activity of the *Bsep* gene. Our previous published results show that RXR is rapidly modified by LPS-induced signaling (i.e., within 1 hour) which reduces most DNA binding; however that may not be the optimal time point for look at for binding and correlation to gene expression. Alternatively, other factors in the transcriptional complex such as coregulators or RNA-Pol-II are still maintained and may be capable to interact with the complex, not requiring the DBD of RXR. However, binding of RXR and FXR was additionally reduced in response to LPS in *hs-Rxr ex4-/-* mice compared to wt mice, suggesting other RXR domains (e.g. mouse Ser265 phosphorylation (32)) in RXR DNA binding are still a necessary component of *Bsep* RNA downregulation by LPS.

In contrast to *Bsep*, binding of RXR and FXR was essential to maintain basal *Ost* transcriptional activity, indicating these are the major regulators of *Ost* transcription. Also in distinction to binding at the *Bsep* promoter, FXR and RXR binding and transcriptional regulation for *Ost* was not influenced by LPS, indicating more complex and gene-specific effects of inflammatory signaling on RXR binding and function. FXR and RXR DNA occupancy of the *Ost* site correlated with each other in wt and *hs-Rxr ex4-/-* mice, but differential binding levels were observed when RXR ligand was present: more RXR binding exceeded FXR binding, potentially hinting at a specific RXR ligand dependent order in regulation of binding.

In sum, these studies using complementary mouse models indicate marked, but gene-specific changes in RXR binding site occupancy in response to LPS-induced signaling. In addition, the expected global responses to RXR missing its DBD were not seen, leading to findings that suggest complex interactions with partners that permit the DBD-deficient RXR protein to be a functional part of the binding complex, likely due to the multiple roles and interactions between NR partners and chromatin. More studies are required to determine the exact nature of the binding potential of the DBD-deficient RXR protein, and how this permits regulation of gene expression and function with other partners and coregulators (7, 18, 32-34).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Ligand pharmaceuticals is thanked for the kind gift of LG268.

References

1. Zollner G, Marschall HU, Wagner M, Trauner M. Role of nuclear receptors in the adaptive response to bile acids and cholestasis: pathogenetic and therapeutic considerations. *Mol Pharm.* 2006; 3:231–251. [PubMed: 16749856]
2. Zollner G, Wagner M, Trauner M. Nuclear receptors as drug targets in cholestasis and drug-induced hepatotoxicity. *Pharmacol Ther.* 2010; 126:228–243. [PubMed: 20388526]
3. Wagner M, Zollner G, Trauner M. Nuclear receptor regulation of the adaptive response of bile acid transporters in cholestasis. *Semin Liver Dis.* 2010; 30:160–177. [PubMed: 20422498]
4. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med.* 1999; 340:448–454. [PubMed: 9971870]
5. Geier A, Fickert P, Trauner M. Mechanisms of disease: mechanisms and clinical implications of cholestasis in sepsis. *Nat Clin Pract Gastroenterol Hepatol.* 2006; 3:574–585. [PubMed: 17008927]
6. Kim MS, Sweeney TR, Shigenaga JK, Chui LG, Moser A, Grunfeld C, Feingold KR. Tumor necrosis factor and interleukin 1 decrease RXRalpha, PPARalpha, PPARgamma, LXRalpha, and the coactivators SRC-1, PGC-1alpha, and PGC-1beta in liver cells. *Metabolism.* 2007; 56:267–279. [PubMed: 17224343]
7. Ghose R, Zimmerman TL, Thevananther S, Karpen SJ. Endotoxin leads to rapid subcellular re-localization of hepatic RXRalpha: A novel mechanism for reduced hepatic gene expression in inflammation. *Nucl Recept.* 2004; 2:4. [PubMed: 15312234]
8. Trauner M, Arrese M, Lee H, Boyer JL, Karpen SJ. Endotoxin downregulates rat hepatic ntcp gene expression via decreased activity of critical transcription factors. *J Clin Invest.* 1998; 101:2092–2100. [PubMed: 9593765]
9. Li D, Zimmerman TL, Thevananther S, Lee HY, Kurie JM, Karpen SJ. Interleukin-1 beta-mediated suppression of RXR:RAR transactivation of the Ntcp promoter is JNK-dependent. *J Biol Chem.* 2002; 277:31416–31422. [PubMed: 12105223]
10. Ghose R, Mulder J, von Furstenberg RJ, Thevananther S, Kuipers F, Karpen SJ. Rosiglitazone attenuates suppression of RXRalpha-dependent gene expression in inflamed liver. *J Hepatol.* 2007; 46:115–123. [PubMed: 17107731]
11. Beigneux AP, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR. The acute phase response is associated with retinoid X receptor repression in rodent liver. *J Biol Chem.* 2000; 275:16390–16399. [PubMed: 10747970]
12. Geier A, Dietrich CG, Voigt S, Ananthanarayanan M, Lammert F, Schmitz A, Trauner M, et al. Cytokine-dependent regulation of hepatic organic anion transporter gene transactivators in mouse liver. *Am J Physiol Gastrointest Liver Physiol.* 2005; 289:G831–841. [PubMed: 15860642]
13. Kim MS, Shigenaga J, Moser A, Feingold K, Grunfeld C. Repression of farnesoid X receptor during the acute phase response. *J Biol Chem.* 2003; 278:8988–8995. [PubMed: 12519762]
14. Lickteig AJ, Slitt AL, Arkan MC, Karin M, Cherrington NJ. Differential regulation of hepatic transporters in the absence of tumor necrosis factor-alpha, interleukin-1beta, interleukin-6, and nuclear factor-kappaB in two models of cholestasis. *Drug Metab Dispos.* 2007; 35:402–409. [PubMed: 17151194]
15. Teng S, Piquette-Miller M. The involvement of the pregnane X receptor in hepatic gene regulation during inflammation in mice. *J Pharmacol Exp Ther.* 2005; 312:841–848. [PubMed: 15456840]
16. Wan YJ, An D, Cai Y, Repa JJ, Hung-Po Chen T, Flores M, Postic C, et al. Hepatocyte-specific mutation establishes retinoid X receptor alpha as a heterodimeric integrator of multiple physiological processes in the liver. *Mol Cell Biol.* 2000; 20:4436–4444. [PubMed: 10825207]
17. Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. *J Biol Chem.* 2002; 277:18793–18800. [PubMed: 11901146]
18. Kosters A, White DD, Sun H, Thevananther S, Karpen SJ. Redundant roles for cJun-N-terminal kinase 1 and 2 in interleukin-1beta-mediated reduction and modification of murine hepatic nuclear retinoid X receptor alpha. *J Hepatol.* 2009; 51:898–908. [PubMed: 19767119]

19. Xu DX, Wang JP, Sun MF, Chen YH, Wei W. Lipopolysaccharide downregulates the expressions of intestinal pregnane X receptor and cytochrome P450 3a11. *Eur J Pharmacol.* 2006; 536:162–170. [PubMed: 16556443]
20. Okuwaki M, Takada T, Iwayanagi Y, Koh S, Kariya Y, Fujii H, Suzuki H. LXR alpha transactivates mouse organic solute transporter alpha and beta via IR-1 elements shared with FXR. *Pharm Res.* 2007; 24:390–398. [PubMed: 17177110]
21. Zhang J, Chalmers MJ, Stayrook KR, Burris LL, Wang Y, Busby SA, Pascal BD, et al. DNA binding alters coactivator interaction surfaces of the intact VDR–RXR complex. *Nat Struct Mol Biol.* 2011; 18:556–563. [PubMed: 21478866]
22. Zhao Q, Chasse SA, Devarakonda S, Sierk ML, Ahvazi B, Rastinejad F. Structural basis of RXR–DNA interactions. *J Mol Biol.* 2000; 296:509–520. [PubMed: 10669605]
23. Pogenberg V, Guichou JF, Vivat-Hannah V, Kammerer S, Perez E, Germain P, de Lera AR, et al. Characterization of the interaction between retinoic acid receptor/retinoid X receptor (RAR/RXR) heterodimers and transcriptional coactivators through structural and fluorescence anisotropy studies. *J Biol Chem.* 2005; 280:1625–1633. [PubMed: 15528208]
24. Lu J, Cistola DP, Li E. Analysis of ligand binding and protein dynamics of human retinoid X receptor alpha ligand-binding domain by nuclear magnetic resonance. *Biochemistry.* 2006; 45:1629–1639. [PubMed: 16460010]
25. Egea PF, Mitschler A, Rochel N, Ruff M, Chambon P, Moras D. Crystal structure of the human RXRalpha ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. *EMBO J.* 2000; 19:2592–2601. [PubMed: 10835357]
26. Bourguet W, Vivat V, Wurtz JM, Chambon P, Gronemeyer H, Moras D. Crystal structure of a heterodimeric complex of RAR and RXR ligand-binding domains. *Mol Cell.* 2000; 5:289–298. [PubMed: 10882070]
27. Bourguet W, Ruff M, Chambon P, Gronemeyer H, Moras D. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. *Nature.* 1995; 375:377–382. [PubMed: 7760929]
28. Bourguet W, Andry V, Iltis C, Klaholz B, Potier N, Van Dorsselaer A, Chambon P, et al. Heterodimeric complex of RAR and RXR nuclear receptor ligand-binding domains: purification, crystallization, and preliminary X-ray diffraction analysis. *Protein Expr Purif.* 2000; 19:284–288. [PubMed: 10873543]
29. Chandra V, Huang P, Hamuro Y, Raghuram S, Wang Y, Burris TP, Rastinejad F. Structure of the intact PPAR-gamma-RXR-alpha nuclear receptor complex on DNA. *Nature.* 2008; 350:350–356. [PubMed: 19043829]
30. Westin S, Kurokawa R, Nolte RT, Wisely GB, McInerney EM, Rose DW, Milburn MV, et al. Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. *Nature.* 1998; 395:199–202. [PubMed: 9744281]
31. Cornelissen J, Fischer M, Sommerdijk N, Nolte RJM. Helical superstructures from charged Poly(styrene)-Poly(isocyanodipeptide) block copolymers. *Science.* 1998; 280:1427–1430. [PubMed: 9603730]
32. Zimmerman TL, Thevananther S, Ghose R, Burns AR, Karpen SJ. Nuclear export of retinoid X receptor alpha in response to interleukin-1beta-mediated cell signaling: roles for JNK and SER260. *J Biol Chem.* 2006; 281:15434–15440. [PubMed: 16551633]
33. Adam-Stitah S, Penna L, Chambon P, Rochette-Egly C. Hyperphosphorylation of the retinoid X receptor alpha by activated c-Jun NH2-terminal kinases. *J Biol Chem.* 1999; 274:18932–18941. [PubMed: 10383391]
34. Bruck N, Bastien J, Bour G, Tarrade A, Plassat JL, Bauer A, Adam-Stitah S, et al. Phosphorylation of the retinoid x receptor at the omega loop, modulates the expression of retinoic-acid-target genes with a promoter context specificity. *Cell Signal.* 2005; 17:1229–1239. [PubMed: 16038797]
35. Plosch T, Kok T, Bloks VW, Smit MJ, Havinga R, Chimini G, Groen AK, et al. Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1. *J Biol Chem.* 2002; 277:33870–33877. [PubMed: 12105210]
36. Lai K, Harnish DC, Evans MJ. Estrogen receptor alpha regulates expression of the orphan receptor small heterodimer partner. *J Biol Chem.* 2003; 278:36418–36429. [PubMed: 12842887]

37. Wagner M, Halilbasic E, Marschall HU, Zollner G, Fickert P, Langner C, Zatloukal K, et al. CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. *Hepatology*. 2005; 42:420–430. [PubMed: 15986414]
38. Kok T, Wolters H, Bloks VW, Havinga R, Jansen PL, Staels B, Kuipers F. Induction of hepatic ABC transporter expression is part of the PPARalpha-mediated fasting response in the mouse. *Gastroenterology*. 2003; 124:160–171. [PubMed: 12512040]
39. Boyer JL, Trauner M, Mennone A, Soroka CJ, Cai SY, Moustafa T, Zollner G, et al. Upregulation of a basolateral FXR-dependent bile acid efflux transporter OSTalpha-OSTbeta in cholestasis in humans and rodents. *Am J Physiol Gastrointest Liver Physiol*. 2006; 290:G1124–1130. [PubMed: 16423920]

Figure 1. Changes in hepatic canalicular transporter RNA expression to inhibition or activation of RXR in response to LPS

Wild-type and *hs-Rxr^{ex4-/-}* mice and C57Bl/6 male mice were given LG268 (30 mg/kg/day for 5 days) or vehicle by gavage. All mice were i.p injected with 0.9% saline or 2 mg/kg LPS for 16 hrs (n=5–7/group). mRNA expression levels were determined by quantitative real-time PCR. *p< 0.05 vs wt/sal, + p< 0.05 vs wt/lps, ^p<0.05 vs veh/sal, #p<0.05 vs veh/lps.

Figure 2. Changes in hepatic basolateral transporter RNA expression to inhibition or activation of RXR in response to LPS

Wild-type and *hs-Rxr ex4-/-* mice and C57Bl/6 male mice were given LG268 (30 mg/kg/day for 5 days) or vehicle by gavage. All mice were i.p injected with 0.9% saline or 2 mg/kg LPS for 16 hrs (n=5-7/group). mRNA expression levels were determined by quantitative real-time PCR. * p< 0.05 vs wt/sal, + p< 0.05 vs wt/lps, ^p<0.05 vs veh/sal, #p<0.05 vs veh/lps.

Figure 3. Changes in hepatic RNA expression of genes involved in bile acid synthesis and Phase I and II detoxification to inhibition or activation of RXR in response to LPS

Wild-type and *hs-Rxr^{ex4}* mice and C57Bl/6 male mice were given LG268 (30 mg/kg/day for 5 days) or vehicle by gavage. All mice were i.p injected with 0.9% saline or 2 mg/kg LPS for 16 hrs (n=5–7/group). mRNA expression levels were determined by quantitative real-time PCR. $p < 0.05$ vs wt/sal, $+ p < 0.05$ vs wt/lps, $^{\wedge} p < 0.05$ vs veh/sal, $\# p < 0.05$ vs veh/lps.

Figure 4A

Figure 4B

Figure 4. Effects of inhibition or activation of RXR in vivo on binding and levels of nuclear RXR protein

Wild-type and *hs-Rxrα-Δexon4*^{-/-} mice were injected by i.p. with 0.9% saline or 2 mg/kg LPS for 1 hr or 16 hrs. n=5–7/group. Male mice were given LG268 (30 mg/kg/day for 5 days) or vehicle by gavage followed by i.p injection of 0.9% saline or 2 mg/kg LPS for 1 hr or 16 hrs. n=5–7/group. A) Nuclear fractions were isolated and binding of nuclear protein to consensus IR-1 binding was determined by EMSA. Sp: specific cold competition (1000x); Nsp: non-specific cold competition (GATA-2,1000x). B) Nuclear fractions were isolated and protein levels of RXR were determined by Western blot.. C) Binding capability of RXR wt and RXR *Δexon4* nuclear fractions as determined by biotin pull-down assays.

Figure 5. Effects of inhibition or activation of RXR in vivo on binding of RXR and FXR to Bsep and Ost promoters in response to LPS

Wild-type and *hs-Rxr^{ex4-/-}* mice and C57Bl/6 male mice were given LG268 (30 mg/kg/day for 5 days) or vehicle by gavage. All mice were i.p injected with 0.9% saline or 2 mg/kg LPS 1 hr. n=2–6/group. DNA occupancy of FXR and RXR was determined by ChIP-q PCR. p< 0.05 vs wt/sal, + p< 0.05 vs wt/lps, ^p<0.05 vs veh/sal, #p<0.05 vs veh/lps. N.S.: Not Significant

Table 1

Gene name		Primer/probe sequences 5'-3'	Reference
Ntcp	Forward primer Reverse primer Probe	ATGACCACCTGCTCCAGCTT GCCTTTGTAGGGCACCTTGT CCTTGGGCATGATGCCTCTCCTC	(35)
Bsep	Forward primer Reverse primer Probe	CTGCCAAGGATGCTAATGCA CGATGGCTACCTTTGCTTCT TGCCACAGCAATTGACACCCTAGTTGG	(35)
Cyp8b1	Forward primer Reverse primer Probe	TTCGACTTCAAGCTGGTCGA CAAAGCCCCAGCGCCT CGACATACCTGTGCCCCCATTAGA	(36)
Mrp3	Forward primer Reverse primer Probe	TCCCACTTTTCGGAGACAGTAAC ACTGAGGACCTTGAAGTCTTGGA CACCAGTGCATTTCGGGCCTATGGC	(10)
Mrp2	Forward primer Reverse primer Probe	GCTGGGAGAAATGGAGAATGTC GACTGCTGAGGGACGTAGGCTA TGGGCATATCACCATCAAGGGCTCC	(10)
Mrp4	Forward primer Reverse primer Probe	TTAGATGGGCCTCTGGTTCT GCCCACAATTCCAACCTTT ACTGCGCTCATCAAGTCCAGGG	(37)
Abcg5	Forward primer Reverse primer Probe	TCAGGACCCCAAGGTCATGAT AGGCTGGTGGATGGTGACAAT CCACAGGACTGGACTGCATGACTGCA	(35)
Cyp7a1	Forward primer Reverse primer Probe	CAGGGAGATGCTCTGTGTTC AGGCATACATGCAAACTCC AATCTGTCATGAGACCTCC TCCCTTCCGTGA	(35)
Abcg8	Forward primer Reverse primer Probe	GACAGCTTCACAGCCACAA GCCTGAAGATGTCAGAGCGA CTGGTGCTCATCTCCCTCCACCAG	(35)
Mdr2	Forward primer Reverse primer Probe	GCAGCGAGAAACGGAACAG GGTTGCTGATGCTGCCTAGTT AAAGTCGCCGTCTAGGCGCCGT	(35)
Atp8b1	Forward primer Reverse primer	CAC ACC AGG ATG GAG AAT CAG A GCC AGG AGC CAG TGA TGA TTA	
Sult2a1	Forward primer Reverse primer Probe	CATCTCTTCTCCAAGTCTTTCTCAGT CAGACACAAGAATATCTCTGGGATT CCAAGGCCAAGGCGATCTATCTCATG	
Ugt1a1	Forward primer Reverse primer	TCTGAGCCCTGCATCTATCTG CCCCAGAGGCGTTGACATA	
Cyp2b10	Forward primer Reverse primer Probe	CAATGGGGAACGTTGGAAGA TGATGCACTGGAAGAGGAAC CTCTGGCCACCATGAGA	
Slco1a1	Forward primer Reverse primer	GTCTTACGAGTGTGCTCCAGAT GTGGTTAATCCAGCAACTGGGA	
Cyp3a11	Forward primer Reverse primer Probe	GGATGAGATCGATGAGGCTCTG CAGGTATTCCATCTCCATCACAGT CCAACAAGGCACCTCCCACGTATGA	(10)
slco1a4	Forward primer Reverse primer Probe	GACGGCTCAGTGTTCATTC CTTCTAGCTGGTCCCTCTT AACGAGCCATAGAGCACATGACTATTAACA	(37)
Mdr1b	Forward primer Reverse primer Probe	GCTGGACAAGCTGTGCATGA TGGCAGAACTAGGCTTCTGCT CTTCCCTCTTGATGCTGGTGTGGAAC	(38)
Ost	Forward primer Reverse primer Probe	GATGCGGCTCCTTGGAATTA GGAGGAACATGCTTGTCATGAC TCCATCCTGGTCTGGCAGTCCTG	(39)

Gene name		Primer/probe sequences 5'-3'	Reference
Ost	Forward primer Reverse primer Probe	TGTTCCAGGTGCTTGTCATCC CCACTGTTAGCCAAGATGGAGAA CCGCCCTGCAGCCTGCCAT	(39)