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Redundant roles for cJun-N-terminal kinase 1 and 2 in interleukin-1β-mediated reduction and modification of murine hepatic nuclear retinoid X receptor α☆

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***Background/Aims*: Retinoid X receptor α (RXRα), the heterodimeric partner for multiple nuclear receptors (NRs), was shown to be an essential target for inflammation-induced cJun-N-terminal kinase (JNK) signaling in vitro. This study aimed to explore the role of hepatic JNK signaling and its effects on nuclear RXRα levels downstream of interleukin-1β (IL-1β) in vivo.**

***Methods*: Effects of IL-1β on hepatic NR-dependent gene expression, nuclear RXRα levels, and roles for individual JNK isoforms were studied in wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice and in primary hepatocytes of each genotype.**

***Results*: IL-1β administration showed a time-dependent reduction in expression of the hepatic NR-dependent genes Ntcp, Cyp7a1, Cyp8b1, Abcg5, Mrp2, and Mrp3. IL-1β treatment for 1 h activated JNK and resulted in both post-translational modification and reduction of nuclear RXRα. In wild-type primary hepatocytes, IL-1β modified and reduced nuclear RXRα levels time dependently, which was prevented by chemical inhibition of JNK as well as by inhibition of proteasomal degradation. Individual absence of either JNK1 or JNK2 did not significantly influence the reduction or modification of hepatic nuclear RXRα by IL-1β both in vivo and in primary hepatocytes.**

***Conclusions*: Functional redundancy exists for JNK1 and JNK2 in IL-1β-mediated alterations of hepatic nuclear RXRα levels, stressing the importance of this pathway in mediating the hepatic response to inflammation.**

***Keywords*: Liver; RXRα; JNK; IL-1β; Inflammation**

1. Introduction

Bacterial products, including lipopolysaccharide (LPS) released by gram-negative bacteria, are primary inducers of the physiologic manifestations of sepsis. By binding to its cognate receptor toll-like receptor-4 (TLR-4), LPS causes the release of inflammatory cytokines from nonparenchymal cells [1–3], which activate intracellular signaling pathways in hepatocytes, thereby inducing broad changes in hepatic gene expression (i.e., the acute phase response [APR]). LPS can also directly activate TLR-4 that is present on hepatocytes [4,5]. In hepatocytes, the negative APR involves downregulation of expression of key transport proteins regulating uptake and secretion of most biliary components, including

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Abbreviations: IL-1β, interleukin-1β; JNK, cJun-N-terminal kinase; LPS, lipopolysaccharide; RXR, retinoid X receptor.

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reduced sinusoidal uptake and canalicular excretion of bile acids by suppression of Na^+/taurocholate cotransporting polypeptide (Ntcp; solute carrier family 10 [sodium/bile acid cotransporter family], member 1 [Slc10a1]) and Abcb11 (ATP-binding cassette, sub-family B (MDR/TAP), member 11), respectively, as well as reduced expression of multiple canalicular transporters including the polyspecific organic cation transporter Mrp2 (Abcc2), the heterodimeric cholesterol transporters Abcg5/g8, and the phospholipid transporter Mdr2 (Abcb4) [6]. Together, this subsequently leads to a reduction in bile flow; accumulation of toxic compounds, including bile acids in liver and serum; and, eventually, liver damage. Many of these genes are regulated by type 2 nuclear receptors (NRs), which are ligand-activated transcription factors that require heterodimerization with retinoid X receptor α (RXRα; nuclear receptor sub-family 2, group B, member 1 [NR2B1]) to fully function. Previous studies in rodents [6–14] indicated that LPS reduced the expression of NR-dependent genes due to decreased binding of regulatory nuclear proteins to their DNA-binding elements, including type 2 NRs. Recent studies from our laboratory have demonstrated reduced hepatic nuclear RXRα protein levels after LPS administration in vivo and IL-1β in vitro, and as a common partner of multiple NRs, this appears to be a major contributor of reduced hepatic gene expression during negative hepatic APR [6,9,15]. Studies in HepG2 cells [15] support a mechanism in which IL-1β-induced signaling resulted in phosphorylation of nuclear RXRα at serine 260, which required activation of c-Jun-N-terminal kinase (JNK) and induced export of the majority of nuclear RXRα to the cytosol for degradation by the proteasome. From these and other studies, it is clear that JNK-dependent pathways are centrally involved in hepatic inflammatory responses [2,16–18].

The liver expresses two JNK genes, Jnk1 and Jnk2, each consisting of two alternative splicing forms, p54 and p46 [19–21]. Individual *Jnk1* and *Jnk2* knockout mice are viable [20,21], whereas the *Jnk1*/*Jnk2* double knockout is not [22]. Several recent studies have demonstrated shared and distinct functions for JNK1 and JNK2 [23–27]. In primary hepatocytes, for example, deoxycholic acid-induced toxicity is mediated via JNK1, whereas JNK2 is protective [26]. Additionally, JNK1 and JNK2 play opposite roles in the development of type 2 and type 1 diabetes, respectively [24,28], in Th1 and Th2 inflammatory responses [21,29–31] and in obesity and hepatic steatohepatitis [32]. It is not known if JNK isoforms play distinct roles in the negative hepatic APR or specifically in mediating IL-1β changes in RXRα function. Given the central role for IL-1β in the hepatic APR [33–35] and the essential roles for RXRα in a wide variety of hepatic functions [2,16,36,37], we aimed to specifically explore roles for JNK1 and JNK2 in the response of the liver to IL-1β, with a focus on nuclear RXRα levels and function.

2. Materials and methods

2.1. Animal experiments

Wild-type C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA, USA) or derived from our own colonies. *Jnk1*$^{+/-}$ mice [20] and *Jnk2*$^{-/-}$ mice [21] were purchased from Jackson laboratory and further bred in the animal facility of Baylor College of Medicine to generate *Jnk1*$^{-/-}$ mice, *Jnk2*$^{-/-}$ mice, and wild-type mice. Mice were maintained in a temperature- and humidity-controlled environment and provided with water and rodent chow ad lib. Murine IL-1β (Biovision, Mountain View, CA, USA), LPS (*Salmonella typhimurium*; Sigma Chemical Co., St. Louis, MO, USA), or 0.9% saline at doses as indicated in the figure legends were given by intraperitoneal injection, and livers were harvested after 1, 4, 8, or 16 h. Male mice aged 8–10 weeks were used for all experiments. All animal protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

2.2. Quantitative real-time polymerase chain reaction

Total RNA was isolated from mouse liver tissue using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Complementary DNA was synthesized using the Stratascript First-Strand reverse-transcriptase polymerase chain reaction (PCR) kit (Stratagene, La Jolla, CA, USA). Real-time quantitative PCR was performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA, USA) using Taqman Universal PCR master mix (Applied Biosystems). Primers and probes were obtained from Sigma Genosys, and sequences are listed as supplemental data in Table 1. Quantitative expression values were extrapolated from standard curves and were normalized to cyclophilin. Data are expressed as relative, and all treatments were compared to the control group, which was set to 1. All data were analyzed by Mann–Whitney test or two-way analysis of variance. A P values <0.05 were considered significant.

2.3. Cell fractionation and immunoblotting

Nuclear and cytosolic fractions were prepared according to Itoh et al. [38] with modifications. Briefly, liver tissue was homogenized with a Dounce homogenizer (Kontes, Vineland, NJ, USA) in cold hypotonic buffer (10 mM of 4-[2-hydroxyethyl]piperazine-1-ethanesulfonic acid [Hepes], pH 7.5; 1.5 mM of magnesium chloride [$MgCl_2$]; 10 mM of potassium chloride [KCl]; 0.5 mM of dithiothreitol [DTT]; 1 mM of sodium fluoride [NaF], 1 mM of sodium orthovanadate [Na_3VO_4], and a protease inhibitor cocktail [Roche Diagnostics, Indianapolis, IN, USA]). Nuclei were isolated by centrifugation for 5 min at 5000 rpm at 4 °C two consecutive times. The supernatant was saved as cytosolic fraction each time. Nuclear extracts were prepared by lysing nuclei in 140 mM of sodium chloride (NaCl); 2 mM of ethylenediaminetetraacetic acid (EDTA); 1% Nonidet P-40; 50 mM of Tris–hydrogen chloride (HCl), pH 7.2; 1 mM of NaF; 1 mM of Na_3VO_4; and a protease inhibitor cocktail. Protein concentrations were determined by bicinchoninic acid (BCA) assay according to the manufacturer's protocol (Pierce, Rockford, IL, USA). Total JNK and phosphorylated JNK (P-JNK) antibodies were from Cell Signaling (Beverly, MA, USA), and antibodies for IκBα and RXRα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. Murine primary hepatocyte cultures

Murine primary hepatocytes were isolated from wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice according to the two-step perfusion procedure using 0.025% collagenase, as previously described [39,40]. Cells were plated at a density of 500,000 cells per well in six-well Primaria plates (Becton and Dickenson, San Diego, CA, USA). Cells were allowed to attach for 3–5 h in Williams E media (Invitrogen) containing 10% fetal bovine serum, penicillin (10,000 U/mL), streptomycin (10,000 μg/mL), gentamycin (50 μg/mL), glutamine (2 mM), insulin (2.5 μg/mL), transferrin

(2.5 μg/mL), sodium selenite (2.5 ng/mL), and glucagon (4 ng/mL). Cells were cultured overnight in serum-free Williams E media supplemented with glutamine and antibiotics. The following morning, cells were treated with 10 μg/mL of murine IL-1β (R&D Systems) in 0.1% bovine serum albumin (BSA) or phosphate buffered saline (PBS) for the duration mentioned in the figure legends and were pretreated with the following compounds: 30 μM of SP600125 (Calbiochem) in dimethyl sulfoxide (DMSO) and 25 μM of SB203580 in DMSO (Calbiochem), both for 30 min; 10 μM of U1026 in DMSO (Calbiochem) and 10 μM of MG132 in DMSO for 60 min; and 1 nM of leptomycin B in methanol (MeOH) for 60 min. Nuclear and cytosolic extracts were isolated, as described above.

3. Results

3.1. Effect of interleukin-1β on hepatic cytokine expression

Several studies have indicated that IL-1β mediates a substantial component of the rodent response to LPS [41,42]. First, a hepatic dose response to IL-1β was established in our model. IL-1β-induced a significant, rapid, and dose-dependent increase of >20-fold and approximately 15-fold, respectively, for hepatic tumor necrosis factor α (TNFα) and IL-1β messenger RNA (mRNA) levels, with maximum increase at 1 h (Fig. 1). This increase was short lived and returned to baseline after 4 h for all doses of IL-1β. Interleukin-6 (IL-6) mRNA levels were also dose-dependently induced, with maximal induction (15-fold) at 4 h after IL-1β administration, returning to baseline by 8 h. A rapid reduction in IκBα (nuclear factor of κ light chain polypeptide gene enhancer in B-cell inhibitor α) protein levels was detected within 1 h of IL-1β treatment with doses as low as 0.5 μg, and levels returned to baseline at 4 and 8 h. These results confirm that murine IL-1β administration induced a hepatic inflammatory response in vivo, albeit at a lower magnitude and shorter duration compared to 2 mg/kg of LPS (Fig. 1E).

3.2. Effect of interleukin-1β on hepatic nuclear receptor-dependent gene expression

Detailed analyses of mRNA levels from a variety of NR-dependent genes involved in hepatobiliary transport revealed time- and dose-dependent responses, while the highest dose of 5 μg of IL-1β was most effective; therefore, only these results are shown. As shown in Fig. 2, expression of Ntcp was maximally decreased by 60% ($P < 0.05$) after 8 h, whereas the expression of another bile acid uptake transporter Oatp2 was not affected by IL-1β at any of the doses and time points studied (Fig. S1). The basolateral bile acid exporter Mrp3 was rapidly decreased by 65% ($P < 0.05$) after 1 h of IL-1β treatment, whereas no significant changes were seen at the other time points. Neither Mrp4 expression nor

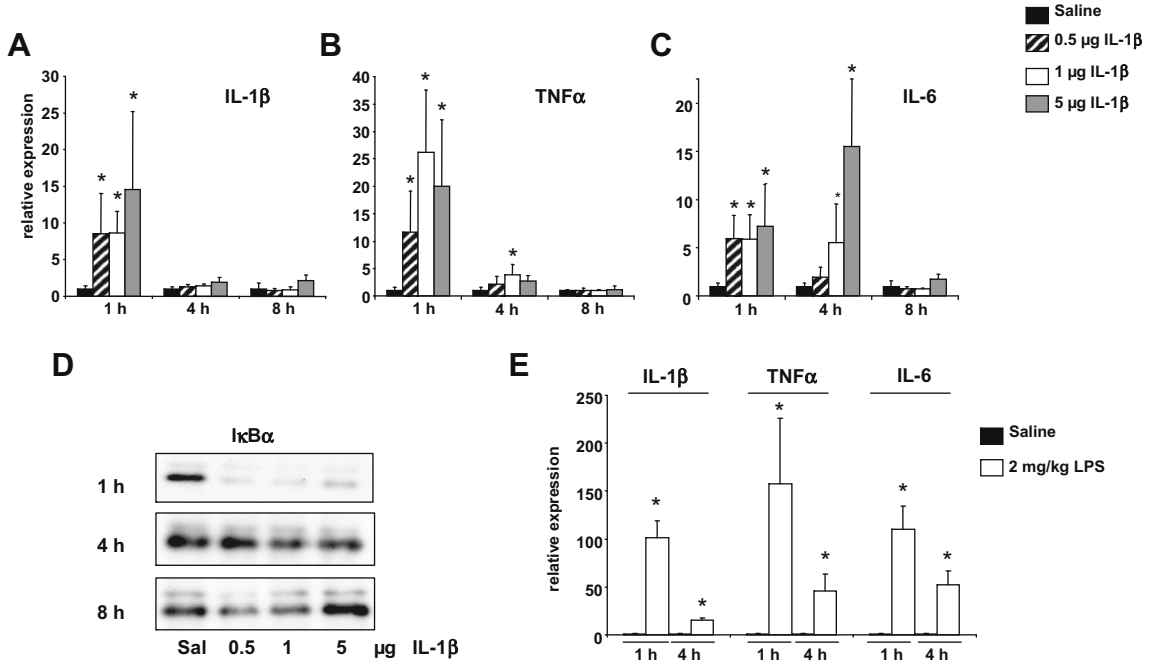

Fig. 1. Interleukin-1β (IL-1β) induced a transient and dose-dependent increase in hepatic cytokine messenger RNA (mRNA) expression and IκBα degradation in mice. Mice were intraperitoneally injected with saline or IL-1β at a dose of 0.5, 1, or 5 μg per 25 g of body weight. Livers were harvested after 1, 4, or 8 h of treatment (five or six per group). Hepatic cytokine expression levels of (A) IL-1β, (B) tumor necrosis factor α (TNFα), and (C) interleukin-6 (IL-6) and (D) protein levels of IκBα degradation were studied as markers to determine IL-1β efficiency in vivo, in comparison to induction by (E) 2 mg/kg of lipopolysaccharide (LPS). Levels of mRNA expression were determined by real-time polymerase chain reaction and normalized to cyclophilin levels. For IκBα (D), samples were pooled from five or six mice per group and IκBα protein levels were determined by Western blot. *Abbreviation:* Sal, saline. *$P < 0.05$ versus saline.

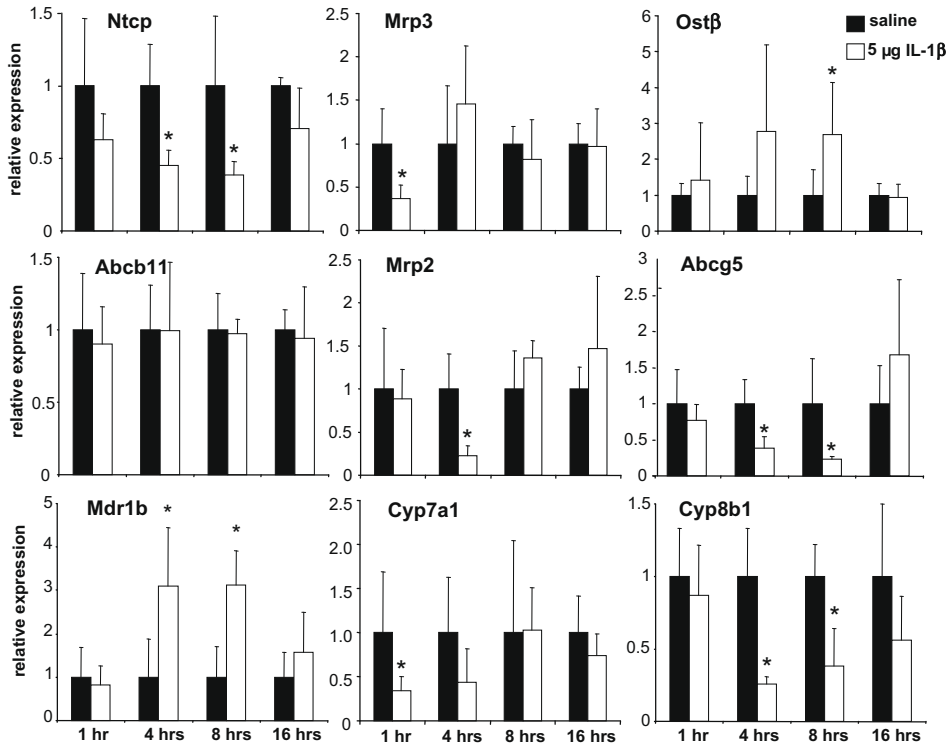

Fig. 2. Interleukin-1β (IL-1β)-induced changes in hepatic nuclear receptor (NR)-dependent gene expression. Mice were injected with 0.9% saline or 5 μg of IL-1β per 25 g of body weight (five or six per group). Livers were isolated after 1, 4, 8, or 16 h. RNA was isolated, and messenger RNA (mRNA) expression levels were determined by Taqman real-time polymerase chain reaction using cyclophilin as the internal control. IL-1β values were compared to saline treatment for each time point. *$P < 0.05$.

organic solute transporter α (Ostα) expression showed any changes at any of the time points (Fig. S1). In contrast, Ostβ expression was upregulated almost 3-fold at 4 and 8 h ($P < 0.05$) after 5 μg of IL-1β. Interestingly, IL-1β administration did not affect Abcb11 expression. Significant changes were observed for mRNA levels of Mrp2 (80% reduced at 4 h of IL-1β) and Abcg5, with a time-dependent reduction of 60% and 77% ($P < 0.05$) at 4 and 8 h, respectively. Mdr1b expression was significantly upregulated 3-fold ($P < 0.05$) at 4 and 8 h after IL-1β administration. Expression of central bile acid synthesis and metabolizing genes were either rapidly downregulated by 65% at 1 h after IL-1β and returned to baseline at 8 h after IL-1β (Cyp7a1), reduced at 4 h (75%) and 8 h (62%) after IL-1β treatment (Cyp8b1), or showed no significant changes in expression (Cyp3a11) (Fig. S1). Altogether, single administration of 5 μg of IL-1β-induced significant but transient changes in hepatic RNA levels of a variety of hepatobiliary transporter and metabolism genes with recovery by 16 h.

3.3. Effect of interleukin-1β on hepatic nuclear retinoid X receptor α and phosphorylated c-Jun-N-terminal kinase

Previous studies from our laboratory found associations between phosphorylation of JNK and reduced nuclear levels of RXRα, both in HepG2 cells after exposure to IL-1β [15] and in livers of wild-type mice after LPS exposure [6]. In the present study, 1 h after exposure to IL-1β, hepatic nuclear RXRα levels were reduced and modified in a dose-dependent manner (Fig. 3A), along with concomitantly increased P-JNK levels in both nuclear and cytosolic compartments. In contrast to IL-1β-treated HepG2 cells and LPS-treated mice, a corresponding increase in cytosolic RXRα was not readily detected in mouse livers at 1 or 4 h after 5 μg of IL-1β (Fig. S2b), suggesting that the actions of IL-1β in mice on nuclear to cytosolic RXRα export occur rapidly or that degradation of RXRα is contained mainly within the nucleus. Nuclear RXRα levels were returned to baseline at 4 h after IL-1β administration (Fig. S2a). Nuclear levels of liver X receptor α (LXRα), farnesoid X receptor (FXR), hepatocyte nuclear factor (HNF) 4α (HNF4α), or HNF1α were not affected by 1 h of 5 μg of IL-1β (Fig. S3). Finally, the duration and degree of suppression of nuclear RXRα levels in response to IL-1β were moderate compared to LPS-induced signaling (Fig. 3B).

3.4. Effect of interleukin-1β on nuclear retinoid X receptor α and phosphorylated c-Jun-N-terminal kinase in murine primary hepatocytes

The liver consists of multiple cell types. To directly link the increase in P-JNK levels to the reduction and

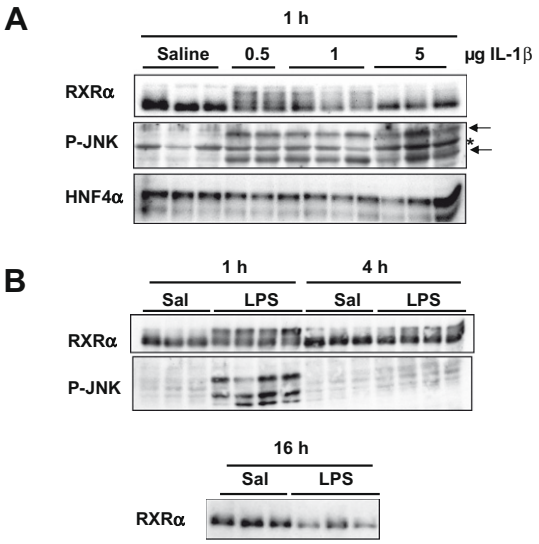

Fig. 3. Interleukin-1β (IL-1β) resulted in increased hepatic levels of phosphorylated cJun-N-terminal kinase (P-JNK) and rapid reduction and modification of hepatic nuclear retinoid X receptor α (RXRα). Wild-type mice were treated with either saline or 0.5 μg, 1 μg, or 5 μg of IL-1β per 25 g of body weight. Livers were isolated after 1 h of treatment. (A) Nuclear fractions were isolated, and protein levels of RXRα and P-JNK were determined by Western blot. (B) Mice were given 2 mg/kg of lipopolysaccharide (LPS) or 0.9% saline by intraperitoneal injections. Livers were harvested 1 h, 4 h, and 16 h later. Nuclear fractions were isolated, and protein levels of RXRα and P-JNK were determined by Western blot. Arrows at P-JNK blots indicate p54 and p46 isoforms. *Abbreviations:* HNF4α, hepatocyte nuclear factor 4 α; Sal, saline. *Indicates nonspecific band. Representative Western blots are shown from five or six mice per treatment group.

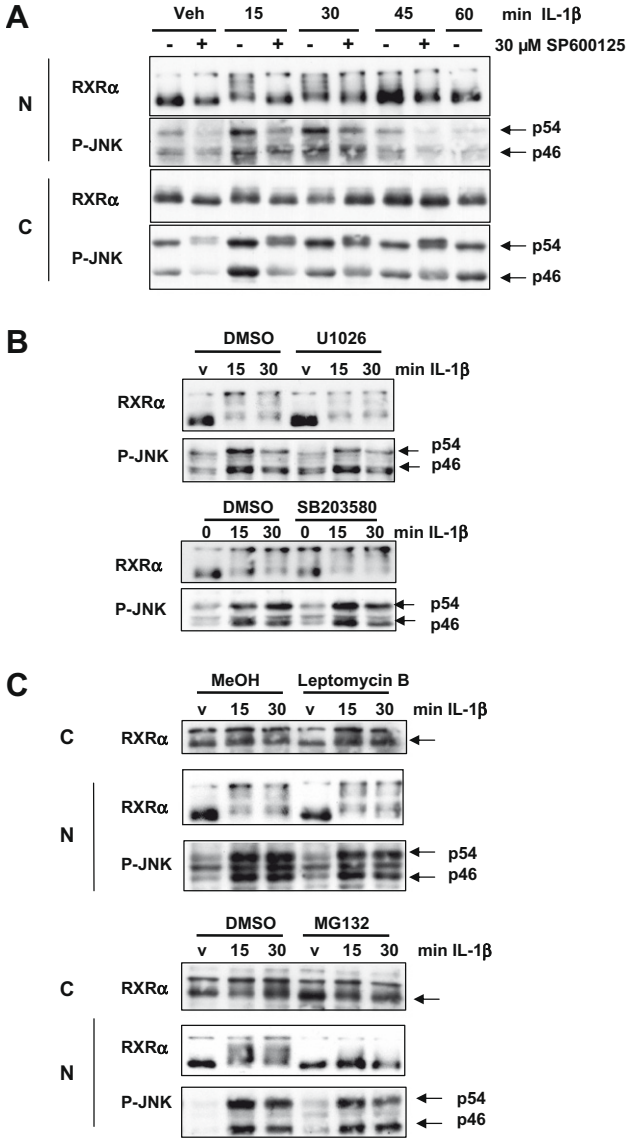

Fig. 4. Interleukin-1β (IL-1β)–mediated reduction and modification of nuclear retinoid X receptor α (RXRα) is dependent on increased phosphorylated cJun-N-terminal kinase (P-JNK) in primary mouse hepatocytes. Primary hepatocytes were serum starved overnight and were treated with 10 ng/mL of murine IL-1β or vehicle (bovine serum albumin [BSA] or phosphate buffered saline [PBS]) for indicated times, with or without 30-minute pretreatment of (A) 30 μM of SP600125 (JNK inhibitor) (+) or (B) 30 μM of SB203580 (p38 inhibitor) or 60-minute pretreatment of 10 μM of U1026 (extracellular regulated kinase pathway inhibitor) or dimethyl sulfoxide (DMSO) (−). (C) Primary hepatocytes were treated with 10 ng/mL of murine IL-1β or vehicle (PBS/BSA) for indicated times, with or without 60-minute pretreatment of 2 nM of leptomycin B (inhibition of nuclear export) or vehicle (methanol [MeOH]) or 10 μM of MG132 (inhibitor of proteasomal degradation) or vehicle (DMSO). Nuclear (N) and cytosolic (C) fractions were isolated, and protein levels of RXRα and P-JNK were determined by Western blot. Representative Western blots are shown from at least five experimental replicates of hepatocytes isolated from different mice.

modifications on nuclear RXRα within the same cell type, murine primary hepatocytes (MPHs) were isolated from wild-type mice and treated with 10 ng/mL of IL-1β for various time points up to 60 min. This resulted in a time-dependent reduction as well as modification of nuclear RXRα as soon as 15 min after treatment, lasting up to 30 min (Fig. 4A). Additionally, the rapid reduction in nuclear RXRα levels, as well as the appearance of several higher molecular weight RXRα species in MPHs after exposure to IL-1β, was prevented by pretreatment with the JNK inhibitor SP600125. This supports a direct role for JNK on mediating post-translational effects of IL-1β on nuclear RXRα. The effects of IL-1β on nuclear RXRα was specifically dependent on JNK activation because inhibition of other signaling pathways downstream of IL-1β such as extracellular regulated kinase 1/2 (ERK1/2; pretreatment with U1026) and p38 mitogen-activated protein kinase (MAPK; pretreatment with SB203580) did not interfere with the effects of IL-1β on RXRα (Fig. 4B). Cytosolic RXRα levels were not affected by either IL-1β or pretreatment of SP600125 in MPHs (Fig. 4A).

To further explore whether nuclear export of RXRα takes place in MPHs in response to IL-1β, cells were pretreated with leptomycin B, an inhibitor of exportin 1 (CRM1)-dependent nuclear export previously shown to inhibit IL-1β-mediated nuclear export of RXRα in HepG2 cells [15]. Fig. 4C shows that nuclear RXRα

in leptomycin B-pretreated cells was reduced and modified in response to 15- and 30-min IL-1β treatment in a manner similar to vehicle-treated hepatocytes. Moreover, pretreatment with leptomycin B did not result in changes in cytosolic RXRα levels, although in MPHs significant amounts of RXRα were found in the cytosol, unlike in vivo. To exclude the possibility that RXRα is immediately degraded after export from the nucleus in response to IL-1β, MPHs were pretreated with the proteasome inhibitor MG132. This did not change cytosolic RXRα levels (Fig. 4C) but instead prevented the reduction and modifications of RXRα in the nuclear compartment. This finding indicates that in MPHs, RXRα is primarily degraded in the nucleus in response to IL-1β rather than relying on export and cytosolic degradation as the main means of regulating nuclear levels of RXRα. Interestingly, MG132 pretreatment did not affect the IL-1β-induced increase in nuclear P-JNK levels.

3.5. Exploration of differential roles for c-Jun-N-terminal kinase 1 and 2

In the results described above, we showed that the effect of IL-1β on nuclear RXRα is mediated by and dependent on intact JNK signaling. To determine whether JNK1 and JNK2 play differential roles in the effect of IL-1β on hepatic gene expression and nuclear RXRα levels, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice were treated with 5 μg of IL-1β for 1 and 4 h, and RNA and nuclear protein levels were evaluated as before. Induction of hepatic IL-1β gene expression by IL-1β treatment was similar in wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice (Fig. 5). In contrast, although the induction of hepatic TNFα and IL-6 RNA levels was not significantly different between wild-type and *Jnk1*$^{-/-}$ mice, there was 47% less and 36% less induction, respectively, in *Jnk2*$^{-/-}$ mice (Fig. 5) ($P < 0.05$). A comparable reduction in gene expression of Ntcp, Cyp8b1, and Abcg5 4 h after IL-1β administration in wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice was observed (Fig. 6A). Additionally, hepatic nuclear RXRα in wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice showed equal levels of reduction and modification 1 h after IL-1β administration (Fig. 6B), indicating functional redundancy of JNK isoforms with respect to mediating the effect of IL-1β on nuclear RXRα and hepatic gene expression. Additionally, in MPHs isolated from *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice, the response of nuclear RXRα to IL-1β could only be inhibited by complete abrogation of JNK activation by pretreatment of SP600125 but not in the hepatocytes lacking each individual *Jnk1* or *Jnk2* gene (Fig. 6C). This shows a direct relationship between IL-1β-mediated JNK activation and the modification and reduction of nuclear levels of RXRα in vivo and in MPHs. Moreover, there appear to be redundant and overlapping roles for JNK1 and

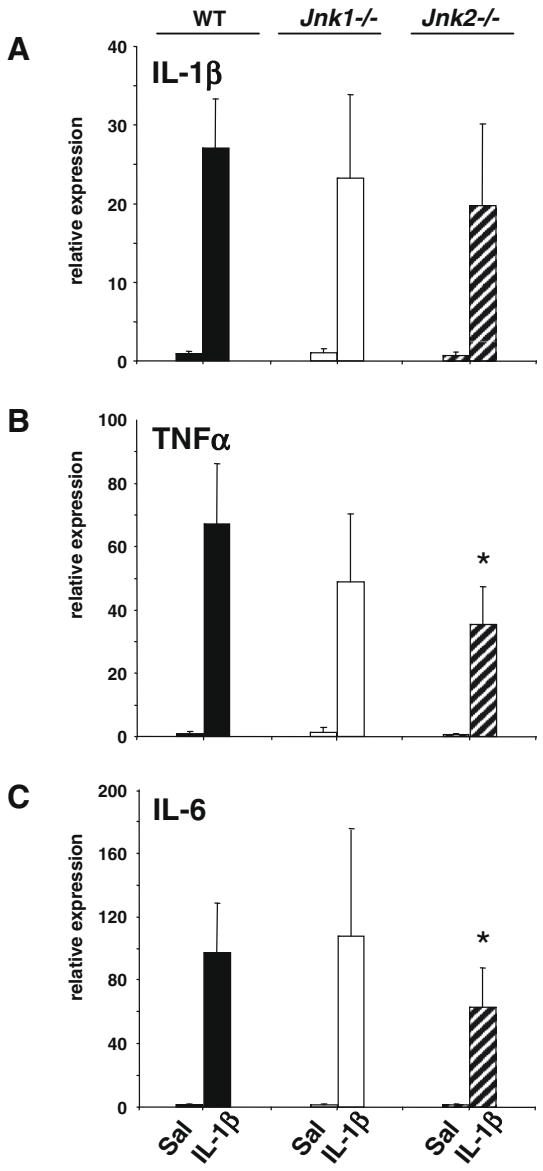

Fig. 5. Interleukin-1β (IL-1β) induces hepatic cytokine messenger RNA (mRNA) expression in wild-type, *Jnk1*$^{-/-}$ and *Jnk2*$^{-/-}$ mice. Mice were intraperitoneally injected with saline or 5 μg of IL-1β per 25 g of body weight prior to isolation of the livers (five or six per group). (A) IL-1β, (B) tumor necrosis factor α (TNFα), and (C) interleukin-6 (IL-6) mRNA expression levels were determined by real-time polymerase chain reaction and normalized to cyclophilin levels and compared to saline-treated wild-type mice. *Abbreviation:* Sal, saline. *$P < 0.05$ versus saline-treated wild-type mice.

JNK2. Because double *Jnk1*/*Jnk2*$^{-/-}$ mice are embryonically lethal, attempts were made to use SP600125 in vivo to pharmacologically inhibit JNK activity completely. This, however, did not result in sufficient inhibition of hepatic JNK activity to modify IL-1β signaling (as determined by phosphorylation of c-Jun) or affect gene expression (data not shown).

Finally, we compared roles for JNK1 and JNK2 in response to 2 mg/kg of LPS. Cytokine levels were equally induced between wild-type, *Jnk1*$^{-/-}$, and

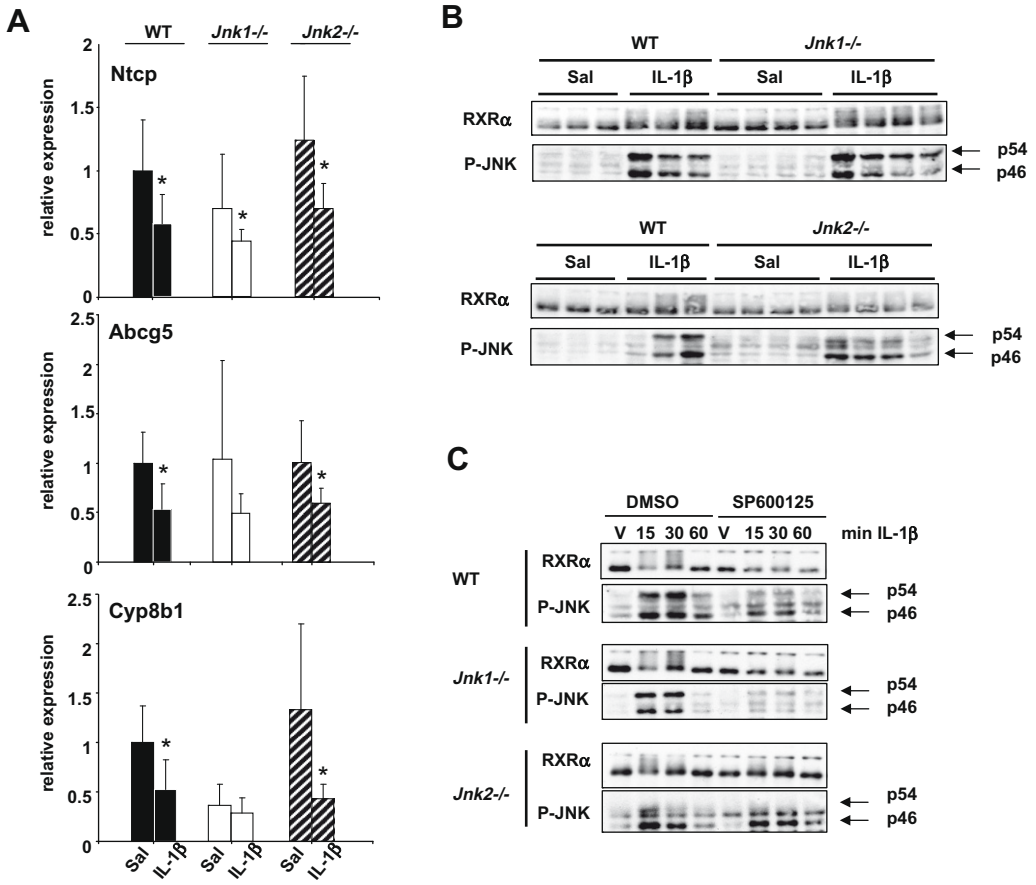

Fig. 6. Redundant roles for cJun-N-terminal kinase (JNK) 1 and 2 in interleukin-1β (IL-1β)–mediated reduction in hepatic gene expression and reduction and modification of nuclear retinoid X receptor α (RXRα). (A) Wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice were intraperitoneally injected with 5 μg of IL-1β or saline 4 hours prior to harvest of the livers. Hepatic RNA was isolated, and messenger RNA expression levels were determined by Taqman real-time polymerase chain reaction (six per group). (B) Wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice were intraperitoneally injected with 5 μg of IL-1β or saline 1 hour prior to harvest of the livers. Nuclear fractions were isolated, and RXRα and phosphorylated JNK (P-JNK) protein levels were determined by Western blot analysis. (C) Primary hepatocytes from wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice were treated with 10 ng/mL of murine IL-1β for the indicated times, with or without 30-minute pretreatment with the JNK inhibitor SP600125 (30 μM) or vehicle (dimethyl sulfoxide [DMSO]). Nuclear fractions were isolated, and protein levels of RXRα and P-JNK were determined by Western blot analysis. Representative Western blots are shown from six mice per group or five experimental replicates for primary hepatocytes. *Abbreviations:* Sal, saline; WT, wild-type.

Jnk2$^{-/-}$ mice after 1 h of LPS (Fig. 7A). Similarly, reduced and modified nuclear RXRα was observed in all genotypes in response to LPS to a similar extent (Fig. 7B). Moreover, no difference between wild-type, *Jnk1*$^{-/-}$, and *Jnk2*$^{-/-}$ mice with respect to gene expression of Abcb11, Ntcp, Mrp2, and Cyp8b1 was observed in response to 16 h of LPS (Fig. 7C).

4. Discussion

Hepatic inflammation induced by LPS from gram-negative bacteria causes a concomitant negative APR characterized by downregulation of hepatic gene expression and disruption of critical physiological processes mediated by the liver, including endobiotic/xenobiotic metabolism, glucose and lipid homeostasis, and bile formation. Many genes regulating these processes are under the control of RXRα and its heterodimeric partners [36,43,44], and reduced binding of several NRs to cognate DNA elements was shown during hepatic inflammation in various models [6,10,11,45]. Therefore, reduced levels and post-translational modification of nuclear RXRα observed under inflammatory conditions in this study and others [6,9–11,15] potentially have wide-ranging consequences for liver function.

One of the main cytokines mediating the effect of LPS is IL-1β [41,42,46]. Several studies have shown that IL-1β mediated reduction of hepatic transporter gene expression in rodents [11,46–48], whereas previous studies from our group [6,8,15] have identified a role for JNK signaling downstream of IL-1β in reducing nuclear RXRα levels and RXRα-dependent gene expression of hepatic transporters.

In this study, we have shown that IL-1β changed the gene-expression levels of the majority of hepatic transporters in mice, although each was affected in a time-specific and dose-dependent manner. To our surprise and in

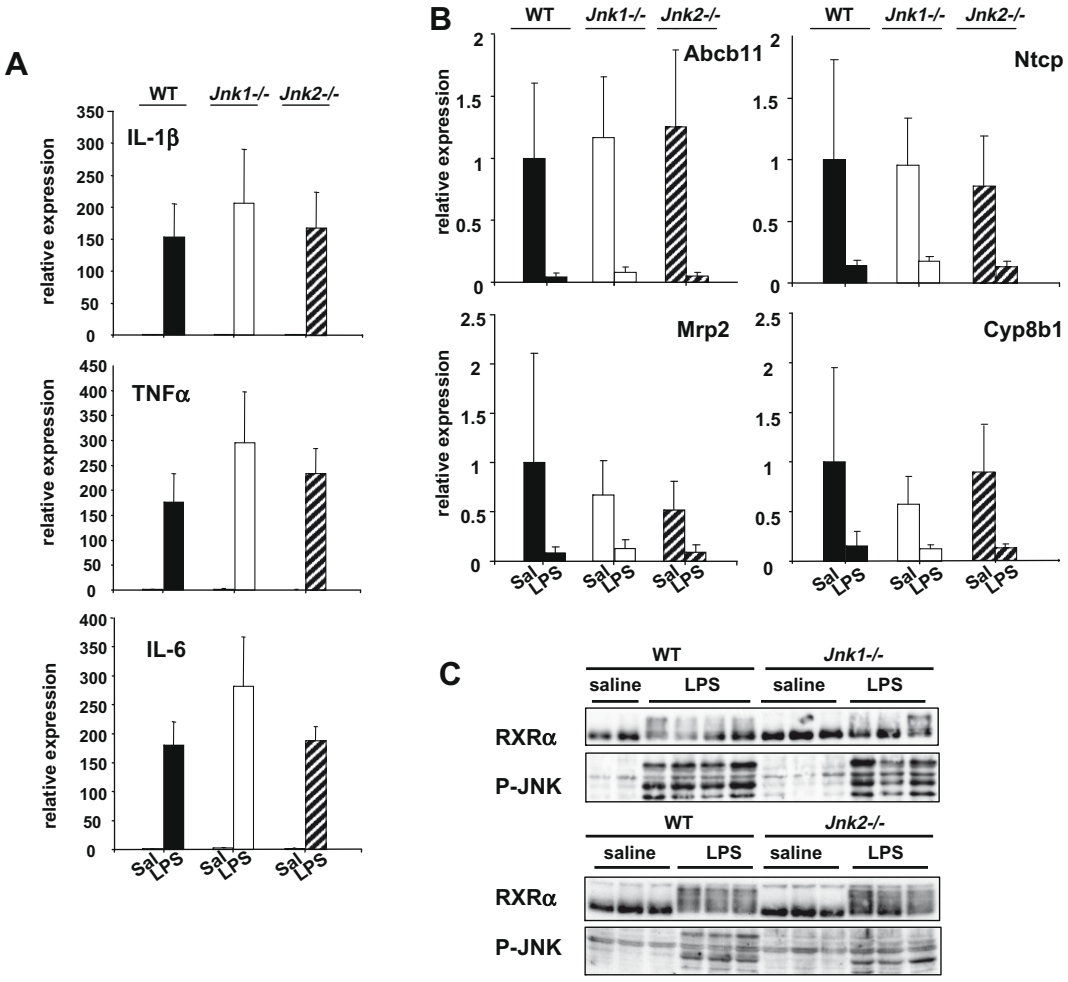

Fig. 7. Redundant roles for cJun-N-terminal kinase (JNK) 1 and 2 in lipopolysaccharide (LPS)–mediated effect on cytokines, hepatic transporter gene expression, and nuclear retinoid X receptor α (RXRα) protein levels. Mice were intraperitoneally injected with 2 mg/kg of LPS or 0.9% saline 1 hour or 16 hours prior to isolation of the livers (five or six per group). (A) Messenger RNA (mRNA) levels of interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), and interleukin-6 (IL-6) were induced after 1 h of exposure to LPS. (B) mRNA levels of Abcb11, Ntcp, MRp2, and Cyp8b1 were determined 16 h after LPS exposure. Expression levels of mRNA were determined by real-time polymerase chain reaction and normalized to cyclophilin levels. (C) Nuclear fractions were isolated, and RXRα and phosphorylated JNK (P-JNK) protein levels were determined by Western blot analysis 1 h after LPS treatment. Representative Western blots are shown from six mice per group. *Abbreviation:* WT, wild type. $^{*}P < 0.05$ versus saline.

contrast with previously published results from other groups [11,47] Abcb11 mRNA expression was not changed by IL-1β administration. These differing results may be explained either by the use of murine IL-1β in this study compared to human IL-1β in others [11], as mature 17-kDa murine and human IL-1β share a sequence identity of only approximately 75%, or by the use of different mouse strains among studies [47]. Additionally, we expand our previous in vitro findings in HepG2 cells [15] and show that IL-1β administration reduced and modified RXRα in nuclear extracts of mouse liver as well as in MPHs treated with IL-1β. P-JNK levels were elevated by IL-1β in both nuclear and cytosolic compartments. These effects of single administration of IL-1β were rapid and short lived, especially in comparison to LPS signaling (Fig. 3). Human RXRα is a known substrate for ERK [49,50] and JNK [8,51] as well as a direct MKK4 target, independent of JNK [52]. In our studies, the effect of IL-1β on nuclear RXRα was mediated strictly via a JNK-dependent mechanism, since inhibition of JNK signaling, but not inhibition of ERK1/2 and P38 MAPK kinase pathways, prevented IL-1β-induced effect on nuclear RXRα in MPHs. A role for MKK4, which is directly upstream of JNK, cannot be completely excluded because the JNK inhibitor SP600125 also inhibits MKK4 [53], albeit with a 10-fold lower affinity.

Although JNK dependency was shown, no distinct role was found for either JNK1 or JNK2, indicating equal capability of each isoform to mediate the effect of IL-1β on RXRα. Additionally, the results obtained with IL-1β reflected the response to LPS in mice but to a more modest degree. This suggests that part of the effects of LPS may be mediated by IL-1β; however, because LPS also induces alterations in expression of many other cytokines, it is likely that multiple cell signaling pathways are involved.

The nature of the rapid modifications of RXRα in response to IL-1β or LPS treatment has not been entirely delineated; however, the observation that it is completely JNK dependent implies that phosphorylation of RXRα is involved. Several JNK phosphorylation sites have been identified in the RXRα protein, spanning all domains [51,54]. Bruck et al. [54] showed that JNK-dependent phosphorylation of murine RXRα at amino acid residues S61, D75, and T87 caused by ultraviolet radiation or anisomycin was responsible for inducing modifications to RXRα on Western blot, similar to our observations in the present study. Consistent with our findings for redundancy of JNK1 and JNK2 in mediating the effect of IL-1β on RXRα, RXRα can be directly phosphorylated by both JNK1 and JNK2 [8,51]. Although stable interactions between JNK and RXRα could not be detected in those studies, our findings of increased P-JNK levels in the nuclear compartment in response to IL-1β supports the possibility of phosphorylation of RXRα by P-JNK either directly or indirectly. Using MPHs, inhibition of proteasome-mediated degradation prevented modification and reduction of nuclear RXRα by IL-1β treatment, despite P-JNK levels remaining elevated. These data may support an indirect role for JNK on RXRα in MPHs, since JNK activation is responsible for both the reduction and the appearance of the high molecular weight forms of nuclear RXRα. Preventing degradation of RXRα by MG132 while JNK is active (Fig. 7) should not influence the modified status of RXRα. Alternatively, direct phosphorylation of RXRα by JNK is inhibited by a protein that is degraded in response to IL-1β, and therefore, MG132 pretreatment would not allow JNK to act on RXRα. Phosphorylation of other NRs has been shown to induce ubiquitination and degradation [55,56], and JNK activates the E3 ligase Itch, necessary for ubiquitination and degradation of the transcription factors cJun and JunB [57]. A similar mechanism may exist to control nuclear RXRα levels. Further studies are necessary to determine the exact nature of interactions between JNK, RXRα, and potential interacting proteins. Nuclear degradation of RXRα in response to IL-1β rather than export and cytosolic degradation also provides an explanation for absence of nuclear export observed in mice.

In summary, we have shown that IL-1β treatment reduced and modified hepatic nuclear RXRα levels in mice. These IL-1β effects required intact JNK signaling; however, redundancy exists for JNK1 and JNK2, which emphasizes the importance of this pathway in mediating hepatic response to inflammation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2009.06.029.

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