Endotoxin Downregulates Rat Hepatic *ntcp* Gene Expression via Decreased Activity of Critical Transcription Factors

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Abstract

Sodium-dependent uptake of bile acids across the hepatic basolateral membrane is rapidly and profoundly diminished during sepsis, thus contributing to the pathogenesis of sepsis-associated cholestasis. This effect is mediated by endotoxin or effector cytokines, which reduce expression of several hepatobiliary transporters, including the sodium-dependent bile acid transporter gene, *ntcp*. We test here the hypothesis that endotoxin treatment leads to impaired binding activity of *ntcp* promoter *trans*-acting factors, resulting in reduction of *ntcp* mRNA expression. After endotoxin administration, *ntcp* mRNA levels reached their nadir by 16 h, and nuclear run-on assays demonstrated a marked reduction in *ntcp* gene transcription. At 16 h after treatment, nuclear binding activities of two key factors that transactivate the *ntcp* promoter, hepatocyte nuclear factor (HNF) 1 and Footprint B binding protein (FpB BP), decreased to 44 and 47% of pretreatment levels, respectively, while levels of the other known *ntcp* promoter transactivator, signal transducer and activator of transcription 5, were unaffected. In contrast, the universal inflammatory response factors nuclear factor κB and activating protein 1 were both upregulated significantly. Examination of nuclear extracts obtained at sequential time points revealed that the maximal decrease in nuclear activities of both HNF1 and FpB BP preceded the nadir of *ntcp* mRNA expression by 6–10 h. Furthermore, these two nuclear factors returned towards normal levels before the recovery of *ntcp* mRNA levels observed by 48 h. Since HNF1α mRNA levels were unchanged at all time points, HNF1 is likely to be regulated posttranscriptionally by endotoxin. We conclude that the downregulation of *ntcp* gene expression by endotoxin is mediated at the level of transcription through tandem reductions in the nuclear binding activity of two critical transcription factors. These findings provide new insight into the coordinated downregulation of hepatobiliary transporters during sepsis. (*J. Clin. Invest.* 1998. 101:2092–2100.) Key words: *ntcp* • bile acid transport • endotoxin • hepatocyte nuclear factor 1 • transcription factors

Introduction

Cholestasis associated with extrahepatic infections and bacteremia has been recognized by clinicians for over one hundred years (1). Septic infants and neonates are particularly affected, but this phenomenon has also been described in older children and adults (2–4). Viral, fungal, and bacterial infections have been linked to cholestasis, although the majority of described cases involve infections with gram-negative bacteria (4). Minor abnormalities in hepatic morphology are frequent (typically Kupffer cell hyperplasia), although the histologic picture is most consistent with intrahepatic cholestasis (4, 5). It has been suggested that these histologic findings are linked to significant perturbations in hepatobiliary transport mechanisms (5).

Endotoxins, which are LPSs contained within the wall of gram-negative bacteria, are well-known stimulants of cytokine production, and the hepatocyte is a pivotal responder to cytokines (for a review, see references 6–8). Infections lead to release of cytokines, and several lines of evidence suggest that LPS-induced cytokines such as TNF-α can lead directly to cholestasis (9). Animal models of endotoxin-induced cholestasis have been developed that reliably reproduce the pathophysiologic changes observed in humans. Significant alterations in both canalicular and basolateral transport systems have been described in the isolated perfused rat liver, rat hepatocytes, and in purified membranes obtained from endotoxin-treated rodents (10–17). Canalicular excretion of conjugated bile acids, bilirubin glucuronides, and glutathione (conjugates) is profoundly decreased after a single dose of endotoxin (12, 13, 16, 17). Moreover, protein and mRNA levels of the canalicular multispecific organic anion transporter (mrp2/cmoat) are downregulated after a single dose of endotoxin, providing a molecular mechanism for the diminished excretion of several non-bile acid organic anions (18). In addition to profound alterations in canalicular transport systems, endotoxin also induces significant changes in sinusoidal transport function, notably by decreasing bile acid uptake (14–16). Under normal conditions, bile acid uptake in hepatocytes is mediated predominantly by the basolateral, sodium-dependent bile acid transport protein (ntcp),[1] which is expressed exclusively in hepatocytes (19). Both ntcp protein and mRNA levels are de-

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1. *Abbreviations used in this paper:* AP, activating protein; APR, acute phase response; FpB, Footprint B; FpB BP, FpB binding protein; HNF, hepatocyte nuclear factor; NF-κB, nuclear factor κB; nt, nucleotide(s); *ntcp*, Na^+/taurocholate cotransporting polypeptide gene; Stat, signal transducer and activator of transcription.

creased significantly after exposure to endotoxin or effector cytokines such as TNF-α or IL-1β (14, 15, 20). The mechanisms underlying the downregulation of *ntcp* gene expression in these models are unknown, but likely involve alterations in regulatory nuclear transcription factors.

The nuclear factors regulating the rat *ntcp* promoter are just beginning to be understood. We have demonstrated recently that expression of the rat *ntcp* promoter in transfected primary rat hepatocyte cultures and HepG2 cells is dependent upon the binding of multiple positive-acting transcription factors. Three *cis*-acting elements within 50 nucleotides (nt) upstream of the mRNA initiation site are essential for basal *ntcp* promoter activity: a TATA element, a hepatocyte nuclear factor (HNF1) binding site, and a binding site for a currently uncharacterized liver-enriched factor, Footprint B (FpB BP) (21). Ganguly et al. have shown that signal transducer and activator of transcription (Stat) 5 activates the *ntcp* promoter via binding to two closely spaced consensus sites located between nt −937 and −904, which explains the postpartum upregulation of *ntcp* expression by prolactin (22).

We hypothesized that the downregulation of rat *ntcp* mRNA levels after endotoxin treatment occurred primarily at the transcriptional level, and that this reduction in *ntcp* gene transcription could be due to alterations in the quantity or function of regulatory nuclear transcription factors. In this study, we show that *ntcp* mRNA levels reached their nadir by 16 h after a single nonlethal dose of endotoxin, followed by a recovery to baseline by 48 h. Nuclear run-on assays indicate that *ntcp* gene transcription is markedly impaired. Interestingly, decreases in the nuclear binding activities of both HNF1 and FpB BP preceded the decline in *ntcp* mRNA levels. Nuclear extracts showed a nuclear accumulation, then fall, of the rapid-responding stress factors nuclear factor κB (NF-κB) and activating protein (AP) 1, while Stat5 levels were essentially unchanged during the entire time course. These data indicate that nuclear binding activities of two critical transactivators of the *ntcp* promoter are specifically reduced after endotoxin challenge, thus providing a mechanism for decreased *ntcp* gene transcription in this cholestatic model of sepsis.

Methods

Materials. TCA-extracted LPS from *Salmonella typhimurium* and sodium salts of taurocholate (> 98% pure by HPLC) were purchased from Sigma Chemical Co. (St. Louis, MO). Molecular biological enzymes and reagents were from Boehringer Mannheim Biochemicals (Indianapolis, IN) and New England Biolabs Inc. (Beverly, MA). [α-^{32}P]deoxycytidine triphosphate, [α-^{32}P]uridine triphosphate, and [γ-^{32}P]adenosine 5′-triphosphate were obtained from Amersham Corp. (Arlington Heights, IL). [^{3}H]Taurocholate was purchased from DuPont-NEN (Boston, MA). The Bradford protein assay was from Bio-Rad Laboratories (Hercules, CA). Cell culture media and FCS were procured from GIBCO BRL (Gaithersburg, MD). All other routine biochemicals were of highest purity commercially available and were purchased from American Bioanalytical, Inc. (Natick, MA), J.T. Baker, Inc. (Phillipsburg, NJ), Bio-Rad Laboratories, Eastman Kodak Co. (Rochester, NY), Fisher Scientific Co. (Pittsburgh, PA), Sigma Chemical Co., and USB Biologicals (Cleveland, OH).

Animals and animal treatment. Male Sprague-Dawley rats (250–275 g) were obtained from Camm Research Institute Inc. (Wayne, NJ). Animals were housed in Plexiglas cages in a temperature- and humidity-controlled room under a constant light cycle and were allowed free access to water and rat chow (Ralston Purina Co., St. Louis, MO). Endotoxemia was induced in rats by intraperitoneal injection with a single, nonlethal dose of LPS from *S. typhimurium* (1 mg/kg body wt) dissolved in sterile 0.9% NaCl (18). LPS in this dose range has been shown previously to induce cholestasis, maximally inhibit bile acid uptake, and significantly reduce *ntcp* mRNA expression 16 h after injection (12, 14–17, 20). Control rats were injected with sterile 0.9% NaCl. 16 h after injection of LPS or vehicle alone, the livers were harvested for preparation of crude liver nuclear extracts after a short perfusion with ice-cold 0.9% NaCl in order to remove intraparenchymal blood. In addition, samples of liver tissue were stored at −70°C until use for isolation of RNA and crude liver membranes. Additional LPS-treated animals were killed for studies of [^{3}H]taurocholate transport (23, 24). In another series of experiments, livers were harvested at different time points (1, 3, 6, 12, 16, 24, and 48 h) after injection of LPS and processed for isolation of RNA and crude nuclear extracts as described below. The study protocols were approved by the Yale Animal Care and Use Committee, and the animals received humane care in compliance with the National Research Council's criteria as outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health (NIH publication 86-23, revised 1985).

Transport studies in isolated rat hepatocytes. The hepatic uptake of taurocholate was assessed using isolated, short-term cultured hepatocytes from control and LPS-treated animals as described by Simon et al. with minor modifications (24). 16 h after treatment of three rats in each group, taurocholate uptake was calculated as the difference between Na^+-containing and Na^+-free buffers. Results are expressed as mean±SD pmol · mg protein^{-1} · 30 s^{-1}.

Preparation of crude liver membranes and Western blot analysis. Crude liver membranes were prepared from freeze-clamped, whole liver tissue as described previously (18). Crude liver membrane preparations (100 μg) were loaded onto a 10% SDS-polyacrylamide gel and subjected to electrophoresis (25). After electrotransfer onto nitrocellulose membranes (Optitran; Schleicher & Schuell, Keene, NH), the blots were blocked with Tris-buffered saline containing 0.1% Tween and 5% dry milk for 1 h at room temperature and incubated overnight at 4°C with the polyclonal rabbit anti-ntcp fusion protein antibody (generously provided by Dr. M. Ananthanarayanan, Mount Sinai School of Medicine, New York) at a dilution of 1:2,000 (26). Immune complexes were detected using horseradish-conjugated goat anti–rabbit IgG F(ab′)$_2$ fragments (dilution 1:1,000) according to the ECL Western blotting kit (Amersham Corp.). Immunoreactive bands obtained by autoradiography were quantified by laser densitometry (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA) and analyzed by ImageQuant software (Molecular Dynamics).

RNA isolation and Northern blot analysis. Total RNA was isolated from freeze-clamped, whole liver tissue by acid guanidinium thiocyanate-phenol-chloroform extraction with subsequent centrifugation in cesium chloride solution (27, 28). Poly A^+ RNA was isolated using the PolyAtract IV kit (Promega Corp., Madison, WI). Total RNA (30 μg) or poly A^+ RNA (2.5 μg) was denatured, electrophoresed on a 1.2% agarose/formaldehyde gel, transferred to a nylon membrane (Genescreen; DuPont-NEN) by overnight capillary blotting, and ultraviolet (UV) cross-linked (UV Stratalinker 1800; Stratagene, La Jolla, CA). Membrane prehybridization, hybridization, and washing procedures were performed as described previously (18). mRNA levels were detected by exposure of the membrane to a PhosphorImager screen (Molecular Dynamics) and quantified using a PhosphorImager and the ImageQuant software. In addition, the blots were exposed to Hyperfilm (Amersham Corp.) for 1–3 d. Differences in mRNA loading were corrected after reprobing the stripped blots for GAPDH. The size of mRNA was estimated by a 0.24–9.5 kb RNA ladder (GIBCO BRL). The specific cDNA probes used were (*a*) *ntcp* cDNA (0.9-kb EcoRI fragment, kindly provided by Drs. B. Hagenbuch and P.J. Meier, University Hospital, Zurich, Switzerland); (*b*) GAPDH cDNA (1.25-kb PstI fragment); (*c*) HNF1α cDNA (3.3-kb BamHI fragment, kindly provided by Dr. Gerald Crabtree, Stanford University School of Medicine, Stanford, CA); and (*d*) rat albumin

cDNA (full-length 2.1-kb fragment, kindly provided by Dr. H. Esumi, National Cancer Center Research Institute, Tokyo, Japan (18, 29–31).

Nuclear run-on transcription assay. Nuclei were isolated immediately after removal of the livers, and aliquots (2×10^7 nuclei) were used to radiolabel total nuclear RNA in the presence of 100 μCi [α-^{32}P]UTP (Amersham Corp.). A modification of the technique described by Gartung et al. was used (32, 33). After labeling nascent nuclear transcripts for 30 min at 30°C, total nuclear RNA was recovered according to the technique of Chomczynski and Sacchi (28), precipitated with isopropanol, and washed twice with 70% ethanol, and residual unincorporated nucleotides were removed by filtration through Sephadex G50 columns (Boehringer Mannheim Biochemicals). After prehybridization in 50% formamide, 4× SSC, 5× Denhardt's solution, 0.1% SDS, 2 mM EDTA, 50 mM Pipes, 100 μg/ml yeast transfer RNA, and 100 μg/ml salmon sperm DNA at 45°C for 24 h, equal amounts of radiolabeled RNA ($5–20 \times 10^6$ cpm/ml) were hybridized at 45°C for 48–72 h to prepared nylon filter strips (Genescreen; DuPont-NEN). Each nylon filter strip contained 5 μg of linearized plasmid DNAs (with inserts of *ntcp* or GAPDH cDNAs, as well as vector pBluescript), which were applied via a BioDot apparatus (Bio-Rad Laboratories). After hybridization, filters were washed twice with 2× SSC for 10 min at 45°C, followed by two washes with 2× SSC/0.1% SDS for 15 min at 37°C, a single wash with 0.1× SSC/0.1% SDS for 15 min at 65°C, and a final rinse in 2× SSC for 5 min at room temperature. The membranes were then exposed to Hyperfilm (Amersham Corp.) for 3–7 d, quantified by laser densitometry (Personal Densitometer SI; Molecular Dynamics), and analyzed by ImageQuant software.

Preparation of nuclei and nuclear protein extraction. Liver nuclei were prepared from normal and LPS-treated animals at the given time points and extracted as described previously (21). Nuclear protein yields were similar in controls and LPS-treated animals.

Electrophoretic gel mobility shift assays. 2–10 μg of nuclear extracts was incubated on ice for 30 min with 2×10^4 cpm of ^{32}P end-labeled oligonucleotide (see Table I) as described previously (21). Oligonucleotides AP-1 and NF-κB were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), while all other oligonucleotides were synthesized at the Keck Biotechnology Center, Yale University School of Medicine. Double-stranded oligonucleotide probes were end-labeled and purified according to standard procedures (21).

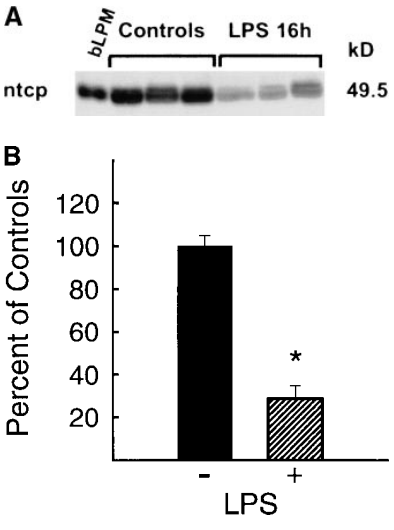

Figure 1. Endotoxin (LPS) reduces ntcp protein levels. Membrane fractions were isolated from control rats (saline vehicle, $n = 4$) and rats 16 h after treatment with LPS (1 mg/kg body wt, $n = 4$), subjected to SDS-PAGE (100 μg protein/lane), and subsequently transferred to nitrocellulose membranes as described in Methods. (*A*) Representative immunoblot. Autoradiographs of three independent samples are shown for each treatment. Molecular weights are given in kilodaltons (kD). *bLPM*, 5 μg of basolateral liver plasma membranes. (*B*) Densitometric analysis of protein levels. Autoradiographs were quantified by laser densitometry, and data (means±SD) are expressed as percentage of controls. *$P < 0.0001$ compared with controls.

In competition assays, 100-fold molar excess of the specific unlabeled oligonucleotide was added to the binding mixtures along with the labeled oligonucleotide. In supershift studies and antibody interference experiments, nuclear extracts were preincubated with 1–2 μl of one of the following antibodies 30 min before the addition of the labeled probe: (*a*) HNF1α polyclonal antibody (the generous gift of Dr. Moshe Yaniv, Pasteur Institute, Paris, France [34]); (*b*) polyclonal antibodies specific for Stat1 (sc-346x), Stat3 (sc-482x), and Stat5 (sc-1656x) (Santa Cruz Biotechnology, Inc.), respectively; and (*c*) polyclonal antibodies specific for jun family members c-*jun* (sc-1694x), junB (sc-73x), and junD (sc-74x) (Santa Cruz Biotechnology, Inc. [35]), respectively. Gels were dried and exposed to autoradiography (Hyperfilm; Amersham, Corp.) at −70° C for 1–3 d. In addition, gels were exposed to a PhosphorImager screen and quantified using a PhosphorImager and the ImageQuant software.

Statistical analysis. Data are expressed as the mean±SD of experiments from four to six animals per group for each time point unless stated otherwise. Calculations of mean values and SD as well as statistical calculations were performed with raw data expressed in relative phosphorimager units. However, for ease of interpretation and comparison of the individual experiments, data are presented graphically as percentage of controls by setting the mean value of the respective controls to 100%. Differences among experimental groups were analyzed by unpaired *t* test or multivariate ANOVA with post-testing when appropriate using the Instat statistic program (GraphPAD Software for Science, San Diego, CA). A P value < 0.05 was considered significant.

Results

Endotoxin (LPS) inhibits expression and function of ntcp. Models of LPS-induced cholestasis vary widely in regard to dose, type, and duration of exposure (14–16, 18, 20). Our model of a low dose, single injection of LPS has been shown to significantly impair canalicular transport systems; however, whether its effects on basolateral bile acid uptake were comparable to previously reported studies was unknown (18). In this study, administration of a single, nonlethal dose of LPS resulted in significant reductions in ntcp function, protein mass, and mRNA levels. 16 h after injection, Na^+-dependent taurocholate uptake in isolated, short-term cultured hepatocytes was reduced by 69% (319.0±92.2 in control rats vs. 100.4±35.2 pmol · mg protein^{-1} · 30 s^{-1} in LPS-treated rats; $P < 0.05$), whereas sodium-independent transport was essentially unaffected (74.4±8.3 vs. 109.3±25.6 pmol · mg protein^{-1} · 30 s^{-1}). Moreover, ntcp protein mass was reduced by 67% (Fig. 1), and steady state *ntcp* mRNA levels were decreased by 86% (Fig. 2). These results are consistent with previous reports and therefore validate this model of LPS-induced cholestasis.

To determine whether the decline in steady state mRNA levels of *ntcp* was caused by decreased transcription, nuclear run-on assays were performed on nuclei isolated from rat livers 16 h after injection of LPS or vehicle alone (Fig. 3). Neither overall gene transcription (as assessed by in vitro incorporation of radiolabeled nucleotides into total nuclear RNA) nor control GAPDH transcription was significantly affected by LPS treatment (data not shown). In contrast, LPS treatment resulted in a marked (84%) decrease in *ntcp* gene transcription compared with control animal livers. These findings support our hypothesis that the decrease in *ntcp* mRNA levels occurs primarily by a reduction in *ntcp* gene transcription.

Endotoxin (LPS) treatment reduces binding activity of HNF1 and FpB BP. Multiple elements in the rat *ntcp* promoter are bound by positive-acting transcription factors: an

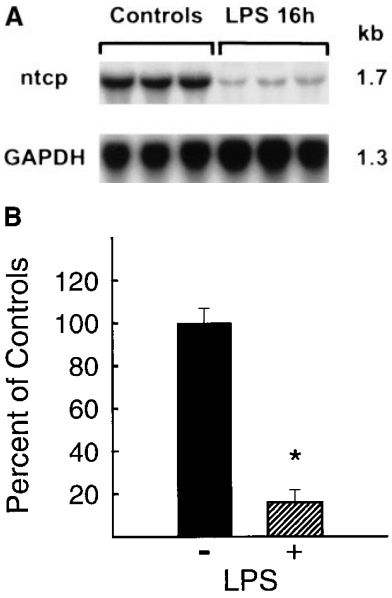

Figure 2. Endotoxin (LPS) reduces *ntcp* steady state mRNA levels. RNA was isolated from control rats (saline vehicle, $n = 4$) and rats 16 h after injection of LPS (1 mg/kg body wt, $n = 4$), and Northern blotting was performed using a radiolabeled probe for *ntcp* as described in Methods. (*A*) Representative Northern blot. Autoradiographs of three independent samples are shown for each treatment. Each lane contains 30 μg of total RNA. Blots were stripped and reprobed for GAPDH to confirm equal loading and RNA integrity. Molecular weights are given in kilobases (kb). (*B*) Quantitative analysis of *ntcp* steady state RNA levels. Blots were quantified by PhosphorImager and normalized individually to GAPDH expression data. Data (means±SD) are expressed as percentage of controls. $^*P < 0.0001$ compared with controls.

HNF1 site at −7/+8, a TATA element at −31/−28, an unknown factor, FpB BP, that binds within FpB (−50/−37), and tandem Stat5 sites (−936/−928 and −912/−904) (Table I, and references 21 and 22). If the LPS-induced decrease in *ntcp* mRNA levels involves a decrease in *ntcp* transcription, and

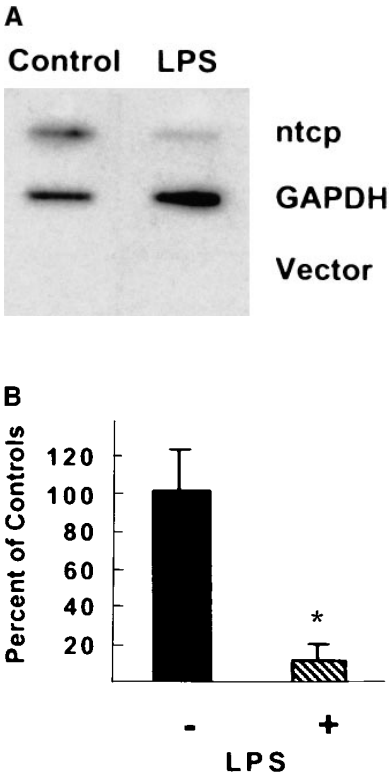

Figure 3. Endotoxin (LPS) decreases transcriptional activity of the *ntcp* gene. Nuclei were isolated from control rats (saline vehicle, $n = 4$) and rats 16 h after injection of LPS (1 mg/kg body wt, $n = 4$) and labeled in the presence of 100 μCi [α-^{32}P]UTP as described in Methods. Total nuclear RNA was isolated and hybridized to membranes containing 5 μg each of the indicated plasmid DNAs. pBluescript plasmid without an insert was used as control. (*A*) Representative autoradiograph. (*B*) Linear densitometer analysis. Data (mean±SD) were expressed as percentage of controls. $^*P < 0.01$ compared with controls.

Table I. DNA Sequences of Double-stranded Oligonucleotides Used for Gel Shift Analyses

Oligonucleotide	Sequence
Footprint A (HNF1)	gatcTGCTGGTTAATCTTTTATTT
−11/+9	ACGACCAATTAGAAAATAAActag
Footprint B	gatcTCCGGGGCATAAGGTTATGG
−56/−37	AGGCCCCGTATTCCAATACCctag
Stat5	gatcTGTCATTCTTGGAAAAATA
−917/−899	ACAGTAAGAACCTTTTTATctag
AP-1	CGCTTGATGACTCAGCCGGAA
	GCGAACTACTGAGTCGGCCTT
NF-κB	AGTTGAGGGGACTTTCCCAGGC
	TCAACTCCCCTGAAAGGGTCCG

transcription is driven by the interplay of sequence-specific transcription factors, then there might be informative alterations in these known *ntcp* transcription factors induced by LPS. Using gel mobility shift assays, we investigated this hypothesis by examining the amounts of these factors in crude liver nuclear extracts prepared 16 h after injection of LPS. Two positive-acting factors, HNF1 and FpB BP, exhibited similar reductions in binding activities (56 and 53%, respectively), whereas Stat5 levels remained essentially unchanged (Fig. 4). Transcription factors known to be activated during the hepatic acute phase response (APR), AP-1 and NF-κB, were increased significantly (324 and 54%, respectively) after LPS administration (Fig. 4). These findings indicate that nuclei prepared 16 h after LPS treatment contain selectively decreased amounts of HNF1 and FpB BP levels, stable amounts of Stat5, and increased amounts of transcription factors AP-1 and NF-κB. For all of the five oligonucleotide probes, the specificity of the DNA–protein interactions was demonstrated by appropriate competition assays. Moreover, supershift experiments revealed that most of the binding activity can be attributed to HNF1α, the predominant isoform in liver (Fig. 5) (36). Since basal *ntcp* gene expression is driven mainly by HNF1 and FpB BP, decreased nuclear binding activity of these essential transcription factors could play an important role in the downregulation of *ntcp* after LPS administration (21).

To exclude the possibility that the lack of demonstrable change in Stat5 binding activity after LPS treatment was due to binding of related LPS-induced Stat proteins, supershift experiments were performed with specific antibodies against Stat1, Stat3, and Stat5, respectively (37). As shown in Fig. 6, in both controls and LPS-treated animals, only preincubation with the specific Stat5 antibody resulted in a supershifted band, demonstrating that related proteins Stat1 or Stat3 do not contribute to the bound complex. Moreover, virtually the entire bound complex was supershifted with Stat5 antibody, indicating that the −917/−899 oligonucleotide probe was recognized exclusively by Stat5.

We were also interested in investigating the identity of proteins that form the AP-1 complex in this model of the hepatic APR. Since several proteins can recognize the same binding site, composed of homo- and heterodimers that typically includes members of the jun family, we were interested in determining if any specific jun family member was involved (38). To analyze the proteins forming the AP-1 complex after administration of LPS, we performed gel shift experiments after prein-

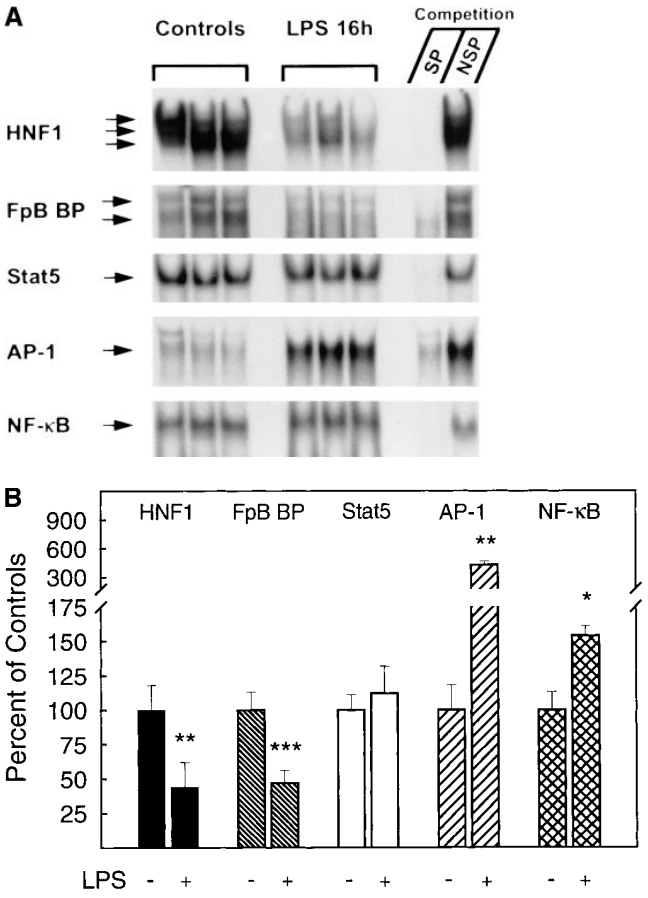

Figure 4. Endotoxin (LPS) specifically inhibits binding activity of HNF1 and FpB BP. Hepatic nuclear extracts were prepared from control rats (saline vehicle, $n = 6$) and rats 16 h after treatment with LPS (1 mg/kg body wt, $n = 6$). 5 μg of crude nuclear extracts was incubated with radiolabeled oligonucleotides representing binding sites for HNF1, FpB BP, Stat5, AP-1, and NF-κB, electrophoresed through a 6% nondenaturing polyacrylamide gel, and autoradiographed, as described in Methods. (*A*) Representative electrophoretic mobility shift assays. Autoradiographs of three independent samples are shown for each treatment. Unlabeled specific (*SP*) and nonspecific (*NSP*) competitor DNAs were included at 100-fold excess and added along with the labeled probe. *Arrows*, The specific bound species. The depicted autoradiographs are overnight (16 h) exposures with the exception of NF-κB (48 h). (*B*) Quantitative analysis of hepatic DNA binding proteins. Gels were quantified by PhosphorImager, and data (means±SD) were expressed as percentage of controls. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$ compared with controls.

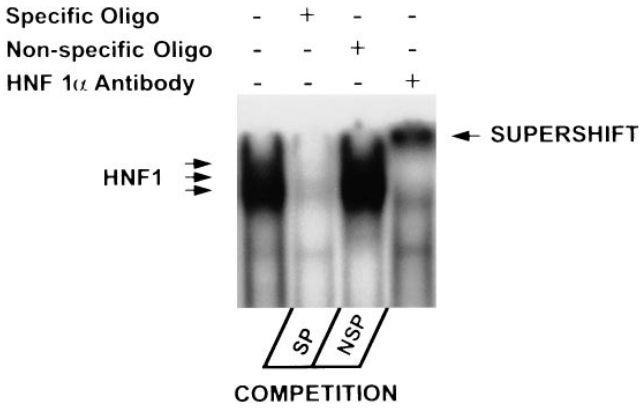

Figure 5. HNF1 binding activity consists primarily of HNF1α. Unlabeled specific (*SP*) and nonspecific (*NSP*) competitor DNAs were included at 100-fold excess and added along with the labeled probe. Nuclear extracts were preincubated with 1 μl (1 mg/ml) of HNF1α polyclonal antibody 10 min before the addition of labeled probe, electrophoresed through a 6% nondenaturing polyacrylamide gel, and autoradiographed as described in Methods. *Arrows*, The specific bound species.

cubation with specific blocking antisera for members of the jun family known to be expressed during the hepatic APR (35, 39). Incubation with antisera that specifically recognize jun family members c-*jun*, junB, or junD each resulted in decreased intensities of the AP-1 complex (Fig. 7). The formation of DNA–protein complexes between hepatic nuclear extracts and the AP-1 binding site probe was unaffected by nonspecific IgG. These results indicate that no single jun family member predominantly contributes to the AP-1 complex, similar to previous findings in another model of the hepatic APR (39).

Endotoxin (LPS) has no effect on HNF1α mRNA expression. To determine whether decreased binding activity of HNF1 was due to decreased expression of this transcription factor, HNF1α steady state mRNA levels were assessed by Northern blotting. In contrast to decreased HNF1 binding activity, LPS treatment did not result in a significant change of HNF1α mRNA levels (Fig. 8). Since the molecular identity of the factor(s) binding within FpB is currently unknown, similar studies could not be performed with FpB BP. These findings indicate that decreased HNF1 binding activity is not caused by reduced HNF1α mRNA expression and suggest that modifications occur at the posttranscriptional level.

Effects of endotoxin (LPS) on ntcp mRNA expression are reversible. Neither the time course of LPS-mediated downregulation of *ntcp* gene expression nor the potential for reversibility has been reported. At various time points up to 48 h after injection of LPS, livers were harvested for RNA isolation. As shown in Fig. 9, *ntcp* mRNA levels decreased rapidly in a time-

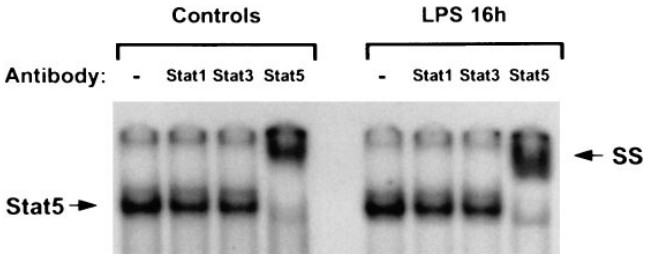

Figure 6. Stat5 binds to the −917/−899 element. Hepatic nuclear extracts were prepared from control rats (saline vehicle) and rats 16 h after receiving LPS (1 mg/kg body wt). 5 μg of crude nuclear extracts was preincubated with 1 μl of specific polyclonal antibodies for Stat1, Stat3, and Stat5, respectively, 10 min before the addition of the radiolabeled oligonucleotide probe, electrophoresed through a 6% nondenaturing polyacrylamide gel, and autoradiographed as described in Methods. *Arrows*, The specific bound species. Note that only addition of the Stat5 antibody results in a supershift (*SS*), whereas antibodies against Stat1 and Stat3 have no effect, confirming that only Stat5 binds to the oligonucleotide probe in both controls and LPS-treated animals.

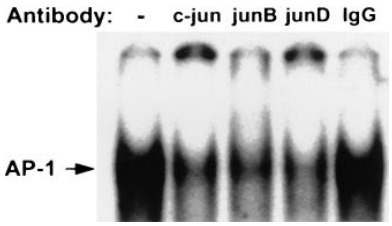

Figure 7. AP-1 complex after endotoxin (LPS) is composed of several jun family members. Hepatic nuclear extracts were prepared from control rats (saline vehicle) and rats 3 h after receiving LPS (1 mg/kg body wt), the time point of maximal AP-1 activation. 2 μg of crude nuclear extracts was preincubated with 2 μl of specific polyclonal antibodies for c-*jun*, jun B, and jun D or nonspecific IgG, respectively, 10 min before the addition of the radiolabeled oligonucleotide probe for AP-1, electrophoresed through a 6% nondenaturing polyacrylamide gel, and autoradiographed as described in Methods. Incubation with antisera specific for c-*jun*, jun B, or jun D interfered with complex formation. Moreover, addition of the c-*jun* and junB-specific antisera repeatedly formed a larger supercomplex retained at the gel origin. Addition of nonspecific IgG had no effect on AP-1 complex formation.

dependent fashion, reaching a nadir between 12 and 16 h after LPS administration and then slowly returning towards normal levels over the following 32–36 h. These findings indicate that the effects of a single dose of LPS on *ntcp* expression are reversible.

Time course of regulatory transcription factor binding activities after endotoxin (LPS) administration. To search for an underlying pattern of hepatic transcription factor binding activities after LPS administration which would support a potential link to the alterations in *ntcp* mRNA levels, mobility shift assays were performed with five oligonucleotide probes (Table I) at various time points after injection of LPS. As shown in Fig. 10, livers from LPS-treated animals contained less HNF1 and FpB BP activity, which was most pronounced

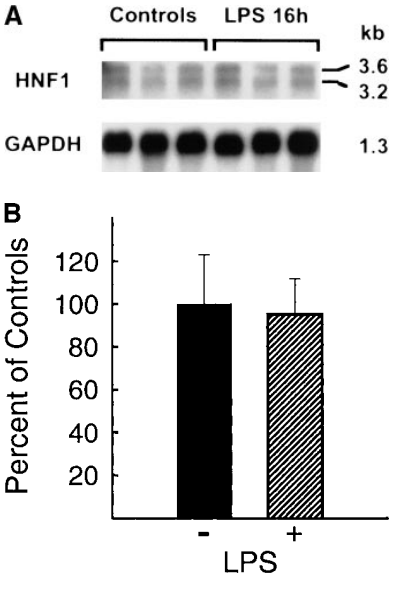

Figure 8. Endotoxin (LPS) does not decrease HNF1α steady state mRNA levels. Poly A^+ RNA was isolated from control rats (saline vehicle, $n = 4$) and rats 16 h after injection of LPS (1 mg/kg body wt, $n = 4$), and Northern blotting was performed using a radiolabeled probe for HNF1α as described in Methods. (*A*) Representative Northern blot. Autoradiographs of three independent samples are shown for each treatment. Each lane contains 2.5 μg of poly A^+ RNA. The blot was stripped and reprobed for GAPDH to confirm equal loading and RNA integrity. Molecular weights are given in kilobases (kb). (*B*) Quantitative analysis of HNF1α steady state RNA levels. Blots were quantified by PhosphorImager and individually normalized to GAPDH expression data. Data (means±SD) are expressed as percentage of controls.

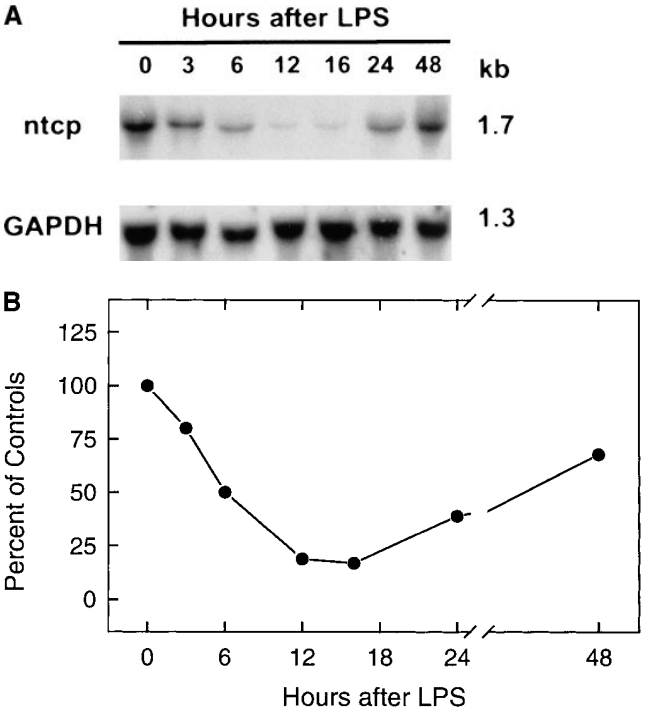

Figure 9. Time course of *ntcp* mRNA levels after endotoxin (LPS) administration. RNA was isolated from rats at various time points after injection of LPS (1 mg/kg body wt), and Northern blotting was performed using a radiolabeled probe for *ntcp*. The blots were stripped and reprobed for GAPDH to confirm equal loading and RNA integrity. (*A*) Representative Northern blot. Each lane contains 30 μg of total RNA. Molecular weights are given in kilobases (kb). (*B*) Quantitative analysis of *ntcp* steady state mRNA levels. Blots were quantified by PhosphorImager and individually normalized to GAPDH expression data. Data ($n = 2$ animals per individual time point) are expressed as percentage of controls. Differences in mRNA loading were corrected by normalization to GAPDH.

between 6 and 16 h after LPS and recovered slowly thereafter. Of note, the nadir of HNF1 and FpB BP activity occurred 6–10 h before the maximal decrease in *ntcp* mRNA levels, further suggesting that the impairment of HNF1 and FpB BP binding activity may play an important role in the LPS-induced reduction of *ntcp* expression. In contrast to HNF1 and FpB BP, Stat5 binding activity remained essentially unchanged throughout the entire time course after LPS exposure. Interestingly, the time course of AP-1 activation coincided with the decrease in HNF1 and FpB BP activity, whereas NF-κB levels peaked and fell before significant changes in the other factors' levels were seen.

Discussion

Recent findings have suggested that endotoxin-associated cholestasis is mediated by reduced activity of hepatobiliary transporters involved in bile formation. Several transporter genes have been cloned, and it has been shown that repression of their function is principally the result of downregulated gene expression (15, 18, 20, 24, 32). Consistent with these published studies, our model of endotoxin-induced cholestasis resulted in a predictable reduction in hepatic Na^+-dependent

taurocholate uptake, ntcp protein mass, and *ntcp* mRNA levels. Nuclear run-on assays indicate that endotoxin treatment decreases *ntcp* mRNA levels by markedly reducing the rate of *ntcp* gene transcription. These results also provide support for a novel mechanism of endotoxin-mediated downregulation of liver gene expression via reduced nuclear binding activities of critical transcriptional activators. We demonstrate that nuclear binding activities of two critical regulators of *ntcp* promoter activity, HNF1 and FpB BP, fall to a minimal level by 6–16 h after a single nonlethal dose of endotoxin and return to baseline levels within 48 h. Moreover, we propose that this pattern of transcription factor regulation directly explains the fall and subsequent restoration of *ntcp* mRNA expression.

HNF1, or more specifically its isoform HNF1α, is one of the key transcription factors regulating genes whose expression defines differentiated hepatocyte function (36). Alterations in HNF1 levels profoundly affect the overall differentiated state of hepatic gene expression in cultured cells, and HNF1α knockout mice show diminished transcription of target genes, including albumin (40, 41). The effect of endotoxin challenge on HNF1 expression has not been extensively addressed. Our experiments indicate that endotoxin treatment leads to reduced quantities of nuclear HNF1 protein. The overall mechanism is posttranscriptional (Fig. 9), suggesting one of several possibilities, including protein modification or enhanced degradation, production of a binding inhibitor, reduced quantities of a necessary binding cofactor, or cytoplasmic trapping (40). Experiments are currently underway to determine the mechanism of this novel finding. In addition to its effects on *ntcp* expression, the reduction in HNF1 binding activity seen in the present studies could also explain endotoxin's suppression of other HNF1-dependent genes, including albumin (see below). We have preliminary evidence that albumin mRNA levels (data not shown) follow a time course similar to *ntcp* mRNA levels after endotoxin administration.

In addition to the effects on HNF1 expression, endotoxin treatment leads to reduced nuclear binding levels of another important, but currently uncharacterized, positive regulator of *ntcp* gene expression, FpB BP. Studies defining the regulation of FpB BP, or the pursuit of any common underlying mechanism that links endotoxin's effects on HNF1 and FpB BP, await cloning of the factor(s) that comprises the FpB BP complex. Stat5 can activate the *ntcp* promoter, yet levels of Stat5 binding activity are unchanged after endotoxin treatment (Fig. 4). It should be noted that the Stat5 elements are not occupied by closely related mediators of the hepatic APR, Stat1 or Stat3 (Fig. 6 [22, 42]). Thus, of the three currently known positive regulators of *ntcp* gene expression, endotoxin specifically downregulates two, HNF1 and FpB BP. We believe that the rapid and profound downregulation of *ntcp* mRNA levels in response to endotoxin challenge is consequent to the combined reduction of the nuclear binding activities of these two important transcriptional activators. Moreover, without adequate nuclear concentrations of these two transactivators, the regulatory protein Stat5 appears to be ineffective in supporting *ntcp* promoter activity.

In response to stimulation by endotoxin, Kupffer cells, the resident liver macrophages, elaborate a host of cytokines, mainly TNF-α, IL-1β, and IL-6, which have been implicated as local, powerful mediators of the hepatic APR (for a review, see references 6, 7, and 43). Hepatocytes respond to these cytokines by producing stereotyped alterations in gene expression, predominantly at the level of gene transcription (44–46). The time course of endotoxin-stimulated cytokine production

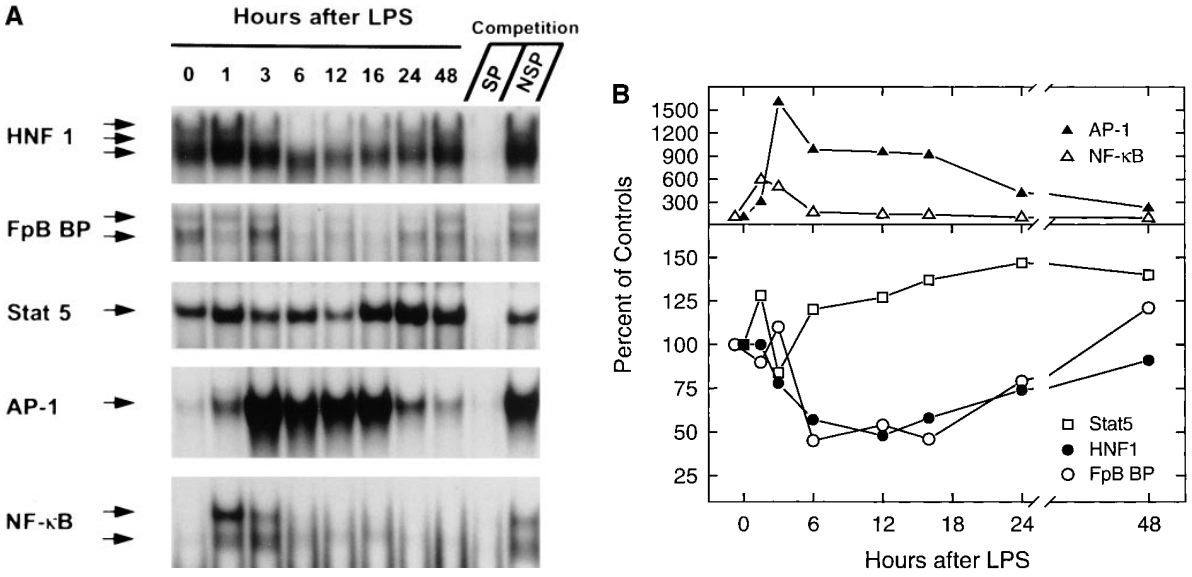

Figure 10. Time course of hepatic DNA binding protein activity after endotoxin (LPS) administration. Hepatic nuclear extracts were prepared from rats at various time points after injection of LPS (1 mg/kg body wt). 5 μg of crude nuclear extracts was incubated with radiolabeled oligonucleotides representing binding sites for HNF1, FpB BP, Stat5, AP-1, and NF-κB, electrophoresed through a 6% nondenaturing polyacrylamide gel, and autoradiographed as described in Methods. (*A*) Representative electrophoretic mobility shift assays. Cold specific (*SP*) and nonspecific (*NSP*) competitor DNAs were included at 100-fold excess and added along with the labeled probe. *Arrows*, The specific bound species. The depicted autoradiographs are 24-h exposures. (*B*) Densitometric analysis of hepatic DNA binding proteins. Gels were quantified by PhosphorImager analysis and expressed as percentage of controls.

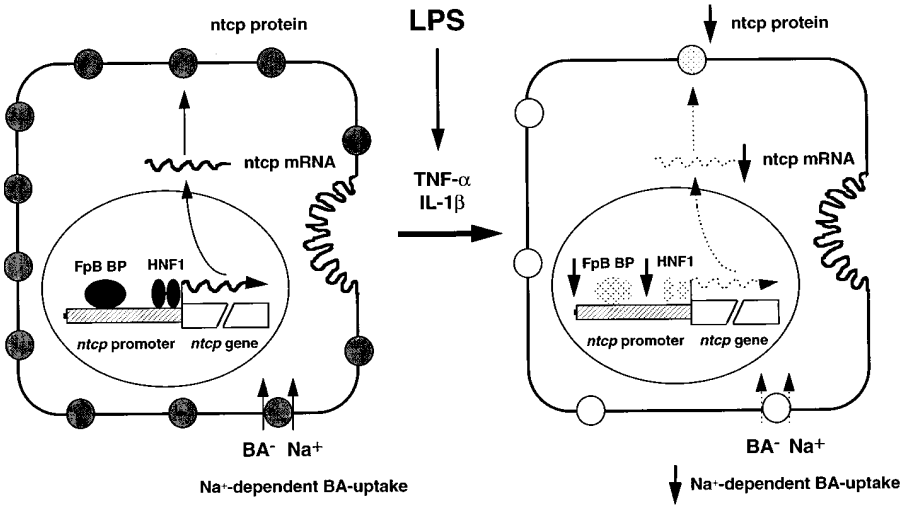

Figure 11. Proposed mechanism for endotoxin's (LPS) effects on *ntcp* gene expression. Schematic representation of hepatocyte before (*left*) and after (*right*) LPS administration. *BA−*, Bile acid. In response to LPS, cytokines are released. Those cytokines reported to induce *ntcp* downregulation, TNF-α and IL-1β, are indicated. The posttreatment hepatocyte (*right*) responds to these mediators with diminished nuclear levels of transactivating factors HNF1 and FpB BP, leading to decreased *ntcp* mRNA expression. In turn, ntcp protein and Na^+-dependent bile acid uptake are reduced markedly. Decreased amounts of transcription factors, mRNA transcripts, and proteins are depicted by faded silhouettes of figures and arrows.

parallels our observed alterations of target transcription factors (47). More direct evidence for the central role played by cytokines in endotoxin-mediated downregulation of *ntcp* mRNA levels has been provided recently by two groups (14, 15, 20). Exposure of rodents or isolated hepatocytes to TNF-α or IL-1β markedly reduced Na^+-dependent bile acid transport and *ntcp* mRNA levels. However, IL-6 had no effect on rat *ntcp* gene expression (20, 48). The available evidence suggests that cytokines such as TNF-α and IL-1β are the likely humoral mediators of endotoxin-induced alterations in hepatic transcription factors observed in the present study. These studies indicate that the *ntcp* gene responds to cytokines in a manner similar to the albumin gene and should be considered as a component of the negative hepatic APR. Albumin mRNA levels fall in response to TNF-α and IL-1β (49, 50). It is quite possible that reduced levels of HNF1 are a common contributing mechanism of the negative hepatic APR. Future studies involving cytokines, signaling inhibitors, and blocking antibodies should help clarify the roles played by each cytokine.

Isolated hepatocytes and hepatocyte-derived cell lines respond rapidly to cytokines by increasing nuclear concentrations of NF-κB and AP-1 (39, 51, 52). Consistent with these studies, we found rapid upregulation of hepatic NF-κB and AP-1 levels after endotoxin challenge, although a direct role for either of these two factors in modulating *ntcp* gene expression is unknown. The nuclear mediators of cytokine induced downregulation of albumin gene expression might include constituents of the AP-1 binding complex (53). Interestingly, the *ntcp* promoter contains potential AP-1 binding sites (21).

In addition to enhancing our understanding of the pathogenesis of sepsis-associated cholestasis, the findings of this study may also have implications for other hepatobiliary disorders associated with increased levels of endotoxin, including alcoholic hepatitis and total parenteral nutrition–induced cholestasis (54). Cholestasis by itself induces bacterial overgrowth in the intestine and facilitates translocation of endotoxin (55). Endotoxin significantly impairs multiple canalicular transport systems, and therefore may "load" the hepatocyte with biliary components, including bile acids (16, 18). Thus, the rapid and profound downregulation of *ntcp* mRNA levels after bile duct ligation could be caused by the combination of retained biliary constituents augmented by the consequences of endotoxemia (32, 56, 57). It should be noted that bile acids can downregulate *ntcp* and cholesterol 7α-hydroxylase promoters (58, 59).

In summary, we provide evidence that endotoxin-mediated reduction of *ntcp* mRNA expression occurs at the transcriptional level, consequent to specific downregulation of two critical regulators of *ntcp* gene expression (see Fig. 11). Furthermore, the reduction of nuclear HNF1 levels likely impacts on the expression of a variety of hepatic genes in endotoxemic states, and might contribute to the negative hepatic APR. Further studies are necessary to define the contribution of retained biliary constituents (e.g., bile acids) and cytokine-stimulated signal transduction pathways on reductions in the nuclear binding activities of HNF1 and FpB BP.

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