

Lipopolysaccharide Results in a Marked Decrease in Hepatocyte Nuclear Factor 4 α in Rat Liver

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The acute-phase response can result in decreased liver-specific functions and death as a result of liver failure. We show here that lipopolysaccharide (LPS), an endotoxin that induces the acute-phase response, results in a marked decrease in the major isoforms of the transcription factor, hepatocyte nuclear factor 4 α (HNF-4 α), in livers of rats. HNF-4 α is a nuclear receptor that is critical for the expression of several liver-specific genes. This decrease in HNF-4 α is primarily the result of a posttranscriptional mechanism, because mRNA levels are normal, and there are no major changes in the splicing patterns. This decrease was of functional significance, because expression of a gene that is highly dependent on HNF-4 α , HNF-1 α , was reduced. Interleukin-1 β (IL-1 β) is a cytokine whose levels are increased *in vivo* in response to LPS. IL-1 β resulted in a decrease in HNF-4 α levels in HepG2 cells. This IL-1 β -induced decrease was likely caused by degradation via the proteasome, because it was prevented by the addition of the proteasome inhibitor, MG132. We conclude that the decrease in HNF-4 α that occurs *in vivo* after the administration of LPS may be the result of IL-1 β -induced degradation, and likely contributes to the liver insufficiency that occurs. IL-1 β antagonists or proteasome inhibitors might increase HNF-4 α protein levels in the acute-phase response, which could result in increased liver function and survival. (HEPATOLOGY 2001;34:979-989.)

The acute-phase response can be induced by bacterial or viral infection, trauma, or other stresses. It can result in liver, kidney, pulmonary, and cardiovascular failure, GI symptoms, and hypotension.^{1,2} The acute-phase response is caused by the production of cytokines that can be activated via a variety of mechanisms. These cytokines bind to receptors on several cell

types and activate signal transduction pathways. Administration of lipopolysaccharide (LPS) results in the production of cytokines such as interleukin-1 β (IL-1 β), IL-6, or tumor necrosis factor α (TNF- α), and stimulates many features of the acute-phase response.

Liver insufficiency after LPS administration is manifested by hypoglycemia, hyperammonemia, hyperbilirubinemia, and coagulopathy,^{3,4} and may be caused by two mechanisms. First, LPS can result in apoptosis of hepatocytes via the TNF- α pathway, which decreases the number of cells.⁵ Second, LPS may decrease hepatic functions in the surviving cells. Indeed, others have demonstrated that LPS decreases CCAAT/enhancer binding protein^{6,7} and hepatocyte nuclear factor 1 (HNF-1)⁸ DNA binding activity in the liver. We demonstrate here that LPS also results in a marked decrease in hepatocyte nuclear factor 4 α (HNF-4 α) protein levels, and that this may contribute to the liver failure that occurs in response to LPS or other inducers of the acute-phase response.

HNF-4 α (NR2A1) is one of the most abundant transcription factors in the liver,⁹ although it is also found in kidney, intestine, stomach, and pancreas. HNF-4 α is a member of the nuclear receptor family, which includes receptors for steroid and thyroid hormones. Most consider HNF-4 α to be an orphan receptor because the activating ligand is unclear,¹⁰ although one group reported that acyl-CoA thioesters of long-chain fatty acids activate HNF-4 α .¹¹ The HNF-4 α gene produces at least 5 isoforms with different transcriptional activity.¹²⁻¹⁸ The exon structure of the isoforms that are known to be expressed in the liver are diagrammed in Fig. 1.

HNF-4 α binds DNA as a homodimer and is critical for the expression of several liver-specific functions. Although homozygous deficiency of HNF-4 α causes embryonic lethality at the time of gastrulation in mice,¹⁹ chimeras that develop with HNF-4 α -deficient livers²⁰ and animals with conditional knock-out of HNF-4 α after birth²¹ are markedly deficient in the expression of several liver-specific genes. Heterozygous deficiency of HNF-4 α is associated with maturity-onset diabetes mellitus of the young 1 in humans²² and a decrease in the expression of some liver-specific genes.²³ The importance of HNF-4 α in the expression of liver-specific genes is further documented by the existence of cell lines that have dedifferentiated as a result of the spontaneous loss of expression of HNF-4 α , and the restoration of differentiation with the transfer of an HNF-4 α gene.²⁴⁻²⁶

The importance of HNF-4 α led us to investigate the mechanism by which LPS results in a marked decrease in its protein levels in rat liver. We found that HNF-4 α mRNA levels are normal, suggesting that a posttranscriptional mechanism is responsible. Because we show here that hepatoma cells exhibited a similar decrease in HNF-4 α in response to IL-1 β , and

Abbreviations: LPS, lipopolysaccharide; IL-1 β , interleukin-1 β ; TNF- α , tumor necrosis factor α ; HNF-4 α , hepatocyte nuclear factor 4 α ; aa, amino acid; RT, room temperature; DR-1, direct repeat 1; nt, nucleotide; ACO, acyl-CoA oxidase; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; ReT, reverse transcriptase.

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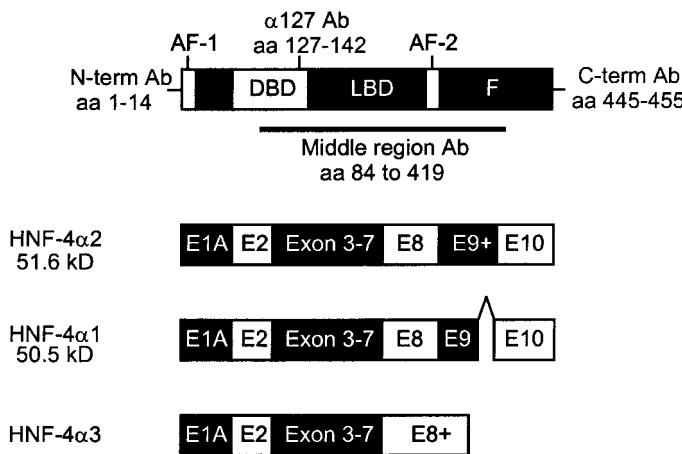


FIG. 1. Diagram of HNF-4 α isoforms. HNF-4 α contains an activation function 1 (AF-1) domain, DNA-binding domain (DBD), ligand-binding domain (LBD), AF-2, and an inhibitory F domain. The amino acids (aa) that were used to generate the N-terminus-specific (N-term Ab), α 127 (α 127 Ab), C-terminus-specific (C-term Ab), and middle-region-specific (Middle region Ab) antibodies are indicated. The HNF-4 α gene encodes at least 5 isoforms that are generated by alternative splicing and/or translational initiation. HNF-4 α 2 is the major isoform in normal liver, it contains 465 aa from 10 exons, and its predicted size based on aa content is 51.6 kd in rat. HNF-4 α 1 has a deletion of aa 410 to 419, resulting in a 455-aa protein. Human HNF-4 α 3 contains a different sequence from aa 369 to the C-terminus as a result of the failure to splice out the intron between exon 8 (E8) and exon 9 (E9), and contains the AF-2 domain, but not the inhibitory F domain. Because an HNF-4 α 3 mRNA has not been reported in rat, the predicted size of the protein it encodes is not indicated.

this could be blocked with a proteasome inhibitor, degradation via the proteasome may be responsible. These results may have implications for the etiology and treatment of liver insufficiency caused by the acute-phase response.

MATERIALS AND METHODS

Reagents. All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated. The rabbit polyclonal anti-HNF-4 α antibody, α 445,¹⁸ that recognizes amino acids (aa) 445 to 455 (PQPTITKQEAI) of rat HNF-4 α 1 is referred to here as the C-terminus antibody. The N-terminus antibody is a rabbit polyclonal raised against a synthetic peptide corresponding to the first 14 aa (MDMADYSAALDPAY) of rat HNF-4 α 1. The middle-region antibody is a rabbit polyclonal raised against aa 84 to 419 of rat HNF-4 α 1 that reacts with several epitopes throughout that region, although they have not been precisely located. The α 127 antibody is a rabbit polyclonal raised against a synthetic peptide corresponding to aa 127 to 142 (RISTRSSYEDSSLPS) of rat HNF-4 α 2. The anti-PPAR α antibodies, N-19X (sc-1985) and C-20X (sc-1982); the anti-RXR α antibody, D-20X (sc-553); and the anti-HNF-4 γ antibody, C-18 (sc-6558), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Animal Procedures. All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985). Male Sprague Dawley rats that weighed 220 to 260 g were treated with LPS from *Escherichia coli* serotype 0111:B4 by intraperitoneal injection as detailed previously.²⁷

Immunoblot Analysis. Nuclear extracts were prepared from rat livers as described in detail,²⁷ and the protein concentration was determined by the Bradford assay. Fifteen micrograms of protein was electrophoresed on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane (Amersham, Cleveland, OH). For the C-terminus antibody, mem-

branes were blocked for 1 hour at room temperature (RT) with TBS-T (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, and 0.1% Triton X-100) with 10% calf serum, and incubated overnight at 4°C in blocking buffer with a 1:2,000 dilution of antibody. For the N-terminus antibody, the membrane was blocked with TBS-T with 1% fat-free dry milk, and incubated with a 1:5,000 dilution of antibody. For the middle-region antibody, the membrane was blocked with TBS-T with 5% fat-free dry milk, and incubated with a 1:500 dilution of antibody. For the α 127 antibody, the membrane was blocked with TBS-T with 1% fat-free dry milk and incubated with a 1:10,000 dilution of antibody. Membranes were incubated with a horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Inc., Piscataway NJ) in the same blocking buffer as was used for the first antibody, and detected by chemiluminescence using Lumi-light Western Blotting Substrate (Boehringer Mannheim, Indianapolis IN). Protein levels were determined by densitometry of the autoradiogram, and normalized to the level found in normal liver after the subtraction of the background signal. Blots were stripped with 62.5 mmol/L Tris (pH 6.8), 2% SDS, and 100 mmol/L β -mercaptoethanol at 65°C for 30 minutes.

Electrophoretic Mobility Shift Assay. The direct repeat 1 (DR-1) binding sites from nucleotides (nt) -67 to -48 of the rat HNF-1 α promoter (GATCTTGA AGTCCA a AGTTCA GTCGATC),²⁸ and nucleotides -574 to -545 of the rat acyl-CoA oxidase (ACO) promoter (GATCCCGGACC AGGACA a AGGTCA CGTTGGGATC)²⁹ were used for the electrophoretic mobility shift assay (EMSA). The sequences shown are that of the top strand after end-labeling. Each hexamer is underlined, and the single nucleotide separating the two hexamers appears in lower case (a). An annealed, double-stranded oligonucleotide probe with a 5' overhang was end-labeled with the Klenow fragment of DNA polymerase I and [α -³²P]dATP. EMSA was performed by incubating 2 μ g of nuclear extract, 10,000 cpm (0.5 ng) of probe, and 0.75 μ g poly dI:dC in 15 μ L of reaction buffer for 30 minutes at RT.²⁷ Samples were electrophoresed on a 4% polyacrylamide gel with 1/2 \times TBE (45 mmol/L Tris-HCl, 45 mmol/L boric acid, 1 mmol/L EDTA) at 4°C. Bands on dried gels were quantified with a phosphorimager (BioRad GS-525, Hercules, CA). Values in samples were normalized to those found in liver nuclear extracts from normal rats that received no injections after subtraction of background, which was the signal present in the corresponding region of the gel for a sample that did not receive any protein. For supershift analyses, 0.1 to 0.2 μ L of antibody was added at the end of this time and incubated for an additional 20 minutes.

Northern Blot Analysis. Twenty micrograms of total RNA prepared from rat livers using guanidinium³⁰ was electrophoresed on a 1% formaldehyde-agarose gel and transferred to an Optitran reinforced nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was hybridized with a full-length rat HNF-4 α probe¹⁸ that was labeled using random primers as per the manufacturer's instructions (Amersham Pharmacia Biotech). To demonstrate that all samples had equal amounts of RNA, blots were stripped with 50% formamide/6 \times SSPE (60 mmol/L sodium phosphate [pH 7.4], 0.9 mol/L NaCl, and 6 mmol/L EDTA) at 65°C for 30 minutes and re-probed with a human 28S rRNA probe.³¹

Immunohistochemical Staining for HNF-4 α . Livers were frozen in OCT, and 8- μ m frozen sections were placed on a slide that was pretreated with 2% saline. The section was fixed for 10 minutes at RT with 3.7% formaldehyde, then blocked with TBS-T with 5% goat serum for 1 hour at RT. Slides were incubated with a 1:200 dilution of the C-terminus-specific antibody in TBS-T with 3% bovine serum albumin at 4°C overnight, washed, and incubated for 1 hour at RT with a horseradish peroxidase-coupled anti-rabbit IgG antibody in TBS-T with 3% bovine serum albumin. The slides were developed for 5 minutes with 3,3'-diaminobenzidine tetrahydrochloride.²⁷

Assay to Determine HNF-1 α mRNA Levels. Nucleotides 156 to 429 of rat HNF-1 α mRNA were amplified by the polymerase chain reaction (PCR) using 1 μ g of RNA from normal rat liver and oligonucleotides that derive from exon 1 (5'-TTCTAAGTTGAGCCAGCTGCA-GACG-3' and 5'-GCTGAGGTTCTCCAGCTTTCAAGA-3'; GI

6981637), and correspond to those that worked well in mouse.²⁰ A reverse-transcriptase (ReT)-PCR reaction was performed with Ready-To-Go PCR beads (Amersham Pharmacia Biotech) and an annealing temperature of 60°C. The PCR product was cloned into pCR-II (Invitrogen Corp., San Diego, CA), and then shuttled into the RNA expression vector, pSP72 (Promega, Madison, WI), as an Eco RI fragment to generate p905. A portion was deleted after digestion with *Apa* I (cuts at nt 256) and *Bst* XI (cuts at nt 341), and religation after blunt-end formation to generate p906. p905 and p906 were restricted with *Bgl* II, which cuts within the polylinker downstream of the coding sequence, and plasmids were transcribed with SP6 RNA polymerase to generate RNAs that resembled a portion of the endogenous mRNA and a shorter competitor, respectively. RNA was precipitated after DNase I digestion, quantified by measuring the optical density at 260 nm, and stored in TE. To determine RNA levels in samples, ReT-PCR was performed with 2 µg of DNase I-treated RNA, 300 fg of the competitor RNA, 0.3 µCi of [α -³²P]-dCTP, the HNF-1 α -specific primers, and Ready-To-Go PCR beads. The ratio of the 274 nt (mRNA) to the 189 nt (competitor) fragment was determined after electrophoresis on an 8-mol/L urea–5% polyacrylamide gel with $\frac{1}{2} \times$ TBE. This was used to calculate the levels of HNF-1 α mRNA after comparison with a standard curve in which 300 fg of the competitor was mixed with varying amounts of the *in vitro*-transcribed longer RNA and ReT-PCR was performed.

Addition of Cytokines to HepG2 Cells. The human hepatocellular carcinoma cell line, HepG2 (ATCC, Rockville, MD), was grown in Dulbecco's modified Eagle medium, supplemented with 10% heat-inactivated fetal bovine serum. Recombinant human TNF- α (GibcoBRL, Rockville, MD) and recombinant human IL-1 β (R&D Systems, Minneapolis, MN) were added at a final concentration of 30 ng/mL and 10 ng/mL, respectively, in complete medium, and cells were incubated at 37°C. For some samples, the proteasome inhibitor, MG-132 (CalBiochem, La Jolla, CA), was added to a final concentration of 10 µmol/L to the medium 1 hour before IL-1 β . Extracts were sonicated for 6 seconds in lysis buffer with 50 mmol/L Tris HCl (pH 8.0); 150 mmol/L NaCl; 1% NP-40; 0.5% deoxycholate; 0.1% SDS; 50 mmol/L NaF; 10 mmol/L β -glycerophosphate; 10 mmol/L Na-molybdate; 5 µg/mL each of aprotinin, pepstatin, and leupeptin; 1 mmol/L phenylmethylsulfonyl fluoride; and 5 mmol/L benzamidine; centrifuged in a microfuge for 20 minutes at 4°C; and 20 µg of protein from the supernatant was analyzed by immunoblot with the C-terminus-specific antibody.

Statistical Analyses. For comparison of levels between normal and LPS-treated rats, the Student *t* test was performed using the program Instat from GraphPAD Software (San Diego, CA).

RESULTS

LPS Results in a Severe Decrease in the Major HNF-4 α Isoforms. The effect of LPS upon HNF-4 α protein levels was determined by performing immunoblot analysis on nuclear extracts prepared from rat livers obtained at various times after injection of LPS. The N-terminus-specific anti-HNF-4 α antibody should recognize all isoforms that contain exon 1A (Fig. 1). The C-terminus-specific antibody should recognize all isoforms with exon 10, but should not recognize HNF-4 α 3. The middle region-specific antibody could recognize sequences from exon 3 to 9, although its exact epitopes have not been characterized. Finally, the α 127 antibody recognizes sequences near the DNA binding domain. As shown in Fig. 2A and quantified in Fig. 2B, the major HNF-4 α isoforms at 52 to 54 kd that were recognized by the N-terminus-specific antibody decreased to <3% of normal at 4.5 hours after LPS in samples from rats that received either 5 mg/kg (lanes 4 and 5) or 15 mg/kg (lanes 12–13). Levels remained low at 10.5 hours (lanes 6–7 and 14–15), but returned to near-normal by 22.5 hours (lanes 8–9 and 16–17). Figure 2C–2E demonstrates that a similar decrease in the 54-kd HNF-4 α isoform was also

observed with the C-terminus-specific, the middle region-specific, and the α 127 antibodies, respectively. The Coomassie staining shown in Fig. 2F demonstrated that these samples had similar amounts of protein.

Although the major isoforms of HNF-4 α fell to very low levels, there were other proteins that were recognized by some, but not all, HNF-4 α antibodies. Figure 2G shows the result when a single membrane was tested sequentially with 3 antibodies to better determine which proteins are recognized by each. The N-terminus-specific antibody recognized a 43-kd protein at all times after administration of LPS that was not recognized by any other antibody, and thus probably represents a nonspecific band. The C-terminus-specific antibody recognized small amounts of proteins that migrated at 37 kd and 35 kd that were not efficiently recognized by any other antibody, and thus may represent nonspecific bands. The middle region-specific and the α 127 antibodies recognized moderate amounts of a 50-kd protein at 1.5 to 10.5 hours after administration of 5 mg/kg of LPS, and lesser amounts of this protein after injection of 15 mg/kg of LPS. Because this is recognized by two different antibodies, it is likely an HNF-4 α isoform or a related protein. In addition, the middle region-specific antibody consistently recognized small amounts of a protein at 47 kd after administration of 5 mg/kg of LPS, and occasionally recognized a protein at 32 kd (Figure 2G, lane 8). Finally, there was an increase in the levels of a 60-kd protein recognized by both the middle region-specific and the α 127 antibodies at 22.5 hours after LPS. This protein was most abundant in samples that received 5 mg/kg of LPS, and was also possibly recognized by the C-terminus-specific antibody (Fig. 2G, lane 12). We conclude that although the 54-kd HNF-4 α isoform is markedly reduced, a smaller isoform that lacks the N- and the C-terminus, or a related protein, remains after LPS.

Effect of LPS on HNF-4 α DNA Binding Activity. HNF-4 α binds to direct repeats of hexamers that resemble AGGTCA and are separated by 1 nucleotide, and thus are referred to as DR-1 sites. To determine the effect of LPS on DNA binding activity, EMSA was performed with two DR-1 site probes. The DR-1 site from the HNF-1 α promoter is reported to be specific for HNF-4 α .²⁸ In contrast, the ACO DR-1 site is somewhat promiscuous, in that it can bind to PPARs, and to a lesser extent, COUP-TFs, in addition to binding to HNF-4 α .^{32,33} As shown in Fig. 3, the overall DNA binding activity fell significantly for both probes after LPS treatment. The major complex present after LPS migrated slightly faster than the complex from normal liver, and is designated as band A. This could contain a smaller protein, such as the 50-kd protein that is recognized on the immunoblot with the middle region-specific and α 127 antibodies. Phosphorimaging demonstrated that the amount of the major complex (the region containing HNF-4 α and band A) was <44% of normal at 1.5 to 10.5 hours after LPS treatment for animals that received 5 mg/kg of LPS ($P < .004$), and <32% of normal for animals that received 15 mg/kg of LPS ($P < .004$). These data demonstrate that the decrease in HNF-4 α protein levels is associated with a decrease in HNF-4 α DNA binding activity, as expected. Another minor, even faster-migrating complex (band B) was observed using the DR-1 site from the ACO promoter, and was increased for some samples from LPS-treated rats. The complete absence of band B using the DR-1 site from the HNF-1 α promoter suggests that this complex is not the result of an HNF-4 α protein.

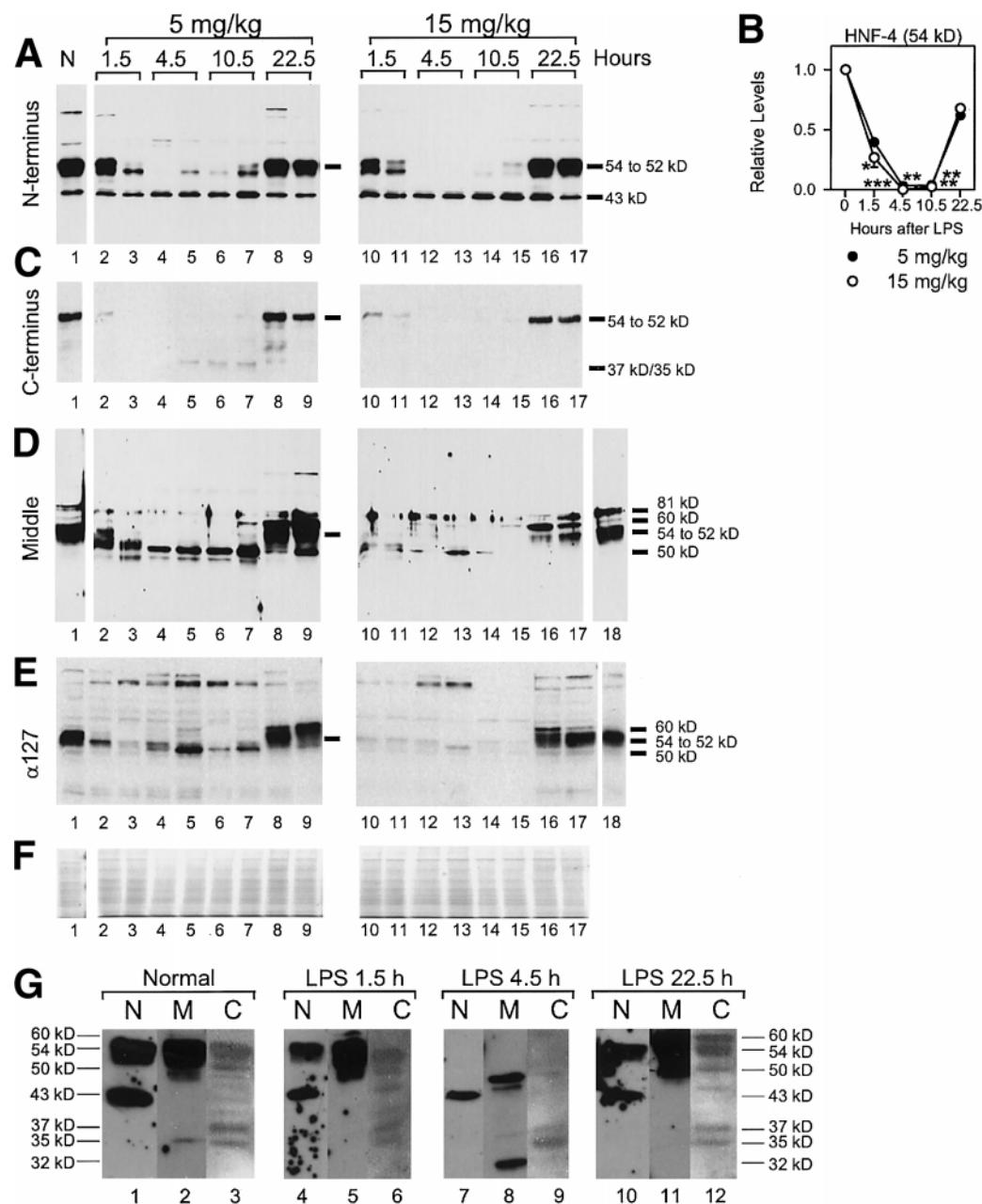


FIG. 2. Immunoblot analysis of HNF-4 α after LPS treatment. Immunoblot analysis was performed on nuclear extracts from livers of normal rats or rats that received LPS. The duplicates for each condition represent samples from 2 different rats that were treated in the same fashion. Similar studies were performed with samples from 2 additional rats at each time point (not shown). (A) N-terminus antibody. Immunoblot analysis was performed with the N-terminus-specific anti-HNF-4 α antibody. Samples were collected from normal rats (N; lanes 1 and 18) or at 1.5, 4.5, 10.5, or 22.5 hours, as indicated, after treatment with 5 mg/kg (lanes 2-9) or 15 mg/kg (lanes 10-17) of LPS. The major isoforms found in the normal liver migrate at a position expected for a 54- to 52-kd protein, which are indicated by a line to the right of lane 9 and lane 17. These are larger than the predicted size of HNF-4 α 1 and HNF-4 α 2, which is presumably the result of a posttranslational modification. An additional band at 43 kd was present, which is likely nonspecific. (B) Quantification of the 52- to 54-kd HNF-4 α protein. Levels of the major isoforms (54- to 52-kd proteins) of HNF-4 α were quantified from the blot shown in (A) and normalized to the level found in normal liver. Values were plotted as the average \pm SEM for each dose of LPS as indicated and compared with those found in normal liver using the Student *t* test. **P* between 0.05 and .005; ***P* between .005 and .0005; ****P* < .0005. (C) C-terminus antibody. Immunoblot was performed as in (A), except for the use of the C-terminus-specific anti-HNF-4 α antibody. In addition to the major isoforms at 54- to 52-kd, low levels of a 37-kd and 35-kd protein were present in some samples. (D) Middle-region antibody. Immunoblot was performed as in (A), except for the use of the middle region-specific anti-HNF-4 α antibody. In addition to the major isoforms at 54 to 52 kd, a protein that migrated at 50 kd was increased at some early times, and a protein that migrated at 60 kd was increased at 22.5 hours. (E) α 127 Antibody. Immunoblot was performed as in (A), except for the use of the α 127 anti-HNF-4 α antibody. Proteins that migrated at 50 and 60 kd were present, as noted in (D). (F) Coomassie staining for evaluation of protein levels. The same amount of protein as that used in (A, C, and D) was electrophoresed and stained with Coomassie to demonstrate that all samples contained similar amounts of protein. The samples that were used for the immunoblot in (E) also had similar amounts of protein after Coomassie staining (not shown). (G) Sequential immunoblot analysis with different anti-HNF-4 α antibodies. Immunoblot analysis was performed using nuclear extracts from normal liver (lanes 1-3), or livers that were collected at 1.5 (lanes 4-6), 4.5 (lanes 7-9), or 22.5 (lanes 10-12) hours after treatment with 5 mg/kg LPS. The same membrane was blotted with the middle region-specific antibody first (lanes 2, 5, 8, and 11), the N-terminus-specific antibody second (lanes 1, 4, 7, and 10), and the C-terminus-specific antibody last (accounting for the high background; lanes 3, 6, 9, and 12). All antibodies recognized the proteins at 54 to 52 kd that are present in normal liver. Only the N-terminus-specific antibody recognized the 43-kd band. The C-terminus antibody also recognized faint bands at 37 kd and 35 kd at most time points, which were not well recognized by the other antibodies. The middle-region antibody recognized bands at 50 kd, 47 kd, and 32 kd at 4.5 hours after LPS (lane 8) that were recognized less efficiently, or not at all, by the other antibodies. The middle-region antibody also recognized a band at 60 kd at 22.5 hours (lane 11) that was also recognized by the C-terminus-specific antibody (lane 12).

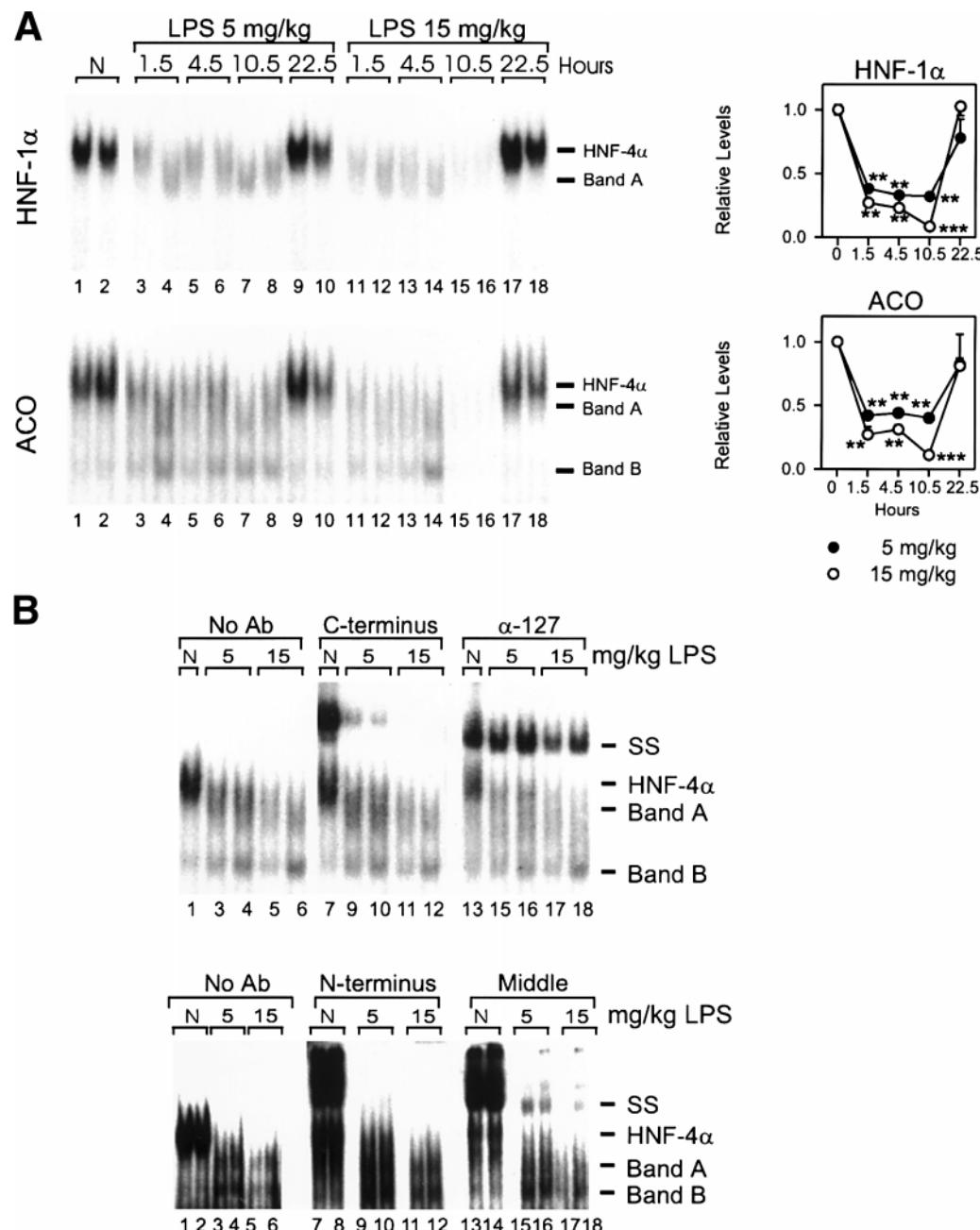


FIG. 3. (A) EMSA with DR-1 sites and liver nuclear extracts after LPS treatment. EMSA was performed using double-stranded oligonucleotide probes from the HNF-1 α and ACO promoters as indicated, and nuclear extracts from normal rats (lanes 1-2) or from rats that were treated with 5 mg/kg (lanes 3-10) or 15 mg/kg (lanes 11-18) of LPS. Samples were collected at 1.5 (lanes 3-4 and 11-12), 4.5 (lanes 5-6 and 13-14), 10.5 (lanes 7-8 and 15-16), or 22.5 (lanes 9-10 and 17-18) hours after treatment. In this and subsequent EMSA, only the complexes that represent binding of protein to probe are shown, but all assays were performed under conditions of excess probe. Duplicates here and in other panels represent samples from 2 different animals that were treated in an identical fashion. The major complex at 4.5 hours after LPS (band A) migrates slightly faster than the complexes present in normal extracts (HNF-4 α). The position of a second complex (band B) is also indicated for the ACO probe. Quantification of the signal from the region of the gel containing both HNF-4 α and Band A relative to that in normal liver are shown on the right. Values were compared with those found in normal liver using the Student *t* test, and criteria for statistical significance are as described in Fig. 2. (B) Supershift analysis of DNA binding using anti-HNF-4 α antibodies. EMSA was performed with the ACO probe and antibodies that recognize HNF-4 α . Samples were from normal livers (lanes 1-2, 7-8, and 13-14) or from livers that were collected from rats at 4.5 hours after treatment with 5 mg/kg (lanes 3-4, 9-10, and 15-16) or 15 mg/kg (lanes 5-6, 11-12, and 17-18) of LPS. The samples were incubated with no antibody (lanes 1-6), the C-terminus-specific antibody (lanes 7-12, top), the α 127 antibody (lanes 13-18, top), the N-terminus-specific antibody (lanes 7-12, bottom), or the middle region-specific antibody (lanes 13-18, bottom). The position of HNF-4 α , band A, and band B are indicated as in (B). The “supershifted” complex (SS) that migrates slower because it contains the antibody in addition to the protein:DNA complex is shown. The data from the top and bottom panels were obtained on different days.

Supershift Analysis to Assess the Specificity of DR-1 DNA Binding Activity. The identity of the proteins present in the complexes observed with the DR-1 sites was further investigated using the ACO probe and supershift analyses with anti-HNF-4 α

antibodies, as shown in Fig. 3B. All of the antibodies could supershift a considerable fraction of the complex from normal liver (lanes 7-8 and 13-14), demonstrating that they recognize HNF-4 α when bound to DNA. Little or no supershift was

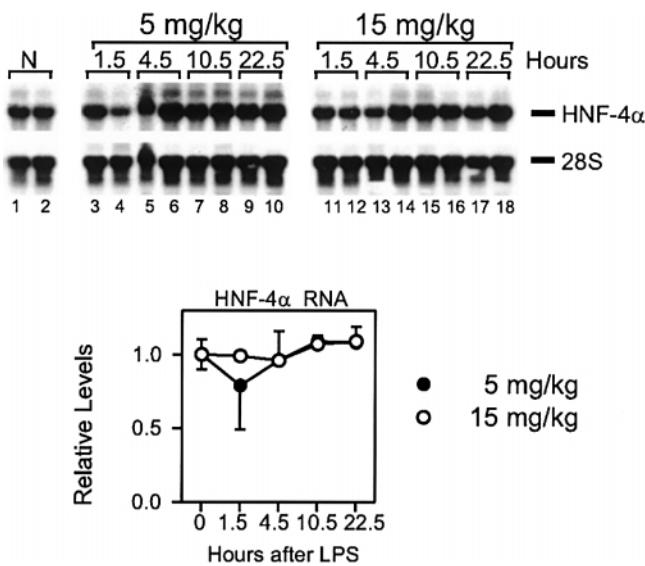


FIG. 4. Analysis of HNF-4 α mRNA after LPS treatment. Northern blot was performed with liver RNA samples and a radiolabeled rat HNF-4 α cDNA probe (HNF-4 α). Samples were from normal liver (lanes 1-2) or from livers of animals that were treated with 5 mg/kg (lanes 3-10) or 15 mg/kg (lanes 11-18) of LPS. The blot was then stripped and reprobed with a 28S rRNA probe (28S) to determine the RNA levels in each sample. Duplicates represent 2 different animals that were treated in an identical fashion. The signal for HNF-4 α mRNA after normalization to 28S rRNA levels relative to that present in normal liver is shown.

observed using samples that were harvested at 4.5 hours after LPS and the anti-HNF-4 α antibodies that recognized the C-terminus (lanes 9-12, top) or the N-terminus (lanes 9-12, bottom) of HNF-4 α . This is consistent with the immunoblot analysis in Fig. 2, which indicates that levels of proteins that contained the C-terminus or the N-terminus of HNF-4 α were very low at this time. The absence of supershift with the N-terminus-specific antibody suggests that the 43-kd protein that it recognized on immunoblot (Fig. 2A) was either a non-specific band or an HNF-4 α protein that does not bind DNA. In contrast, the α 127 antibody resulted in a supershifted band with samples from LPS-treated rats (lanes 15-18, top), suggesting that the extracts contain an HNF-4 α protein with aa 127 to 142 that can bind DNA. The fact that the signal is stronger for the supershifted band than for the original complex may reflect the ability of the antibody to stabilize the protein:DNA complex. Because the level of band A was decreased in the supershift assay with the α 127 antibody (lanes 15-18, top), an HNF-4 α protein is likely to be present in that complex. The middle-region antibody also supershifted at least a portion of band A (lanes 15-18, bottom). None of the anti-HNF-4 α antibodies affected levels of band B, suggesting that it does not contain an HNF-4 α protein. None of the bands were supershifted with antibodies directed against COUP-TFI/ARP-1, PPAR α , or RXR α (data not shown).

Analysis of HNF-4 α mRNA. The decrease in the major isoforms of HNF-4 α in response to LPS could be the result of a decrease in mRNA levels or to other mechanisms. However, the Northern blot analysis shown in Fig. 4 demonstrated that there was no change in the levels or size of the HNF-4 α mRNA. In addition, RT-PCR demonstrated that mRNAs with the normal splicing patterns remained after LPS, as will be summarized in the discussion.

Immunocytochemistry to Determine the Intracellular Location of HNF-4 α . A recent study demonstrated that deacetylation resulted in the failure of HNF-4 α to localize exclusively to the nucleus.³⁴ We therefore tested the hypothesis that the decrease in HNF-4 α in nuclear extracts in response to LPS might result from a redistribution of HNF-4 α , rather than a decrease in protein levels, by performing immunocytochemistry. As shown in Fig. 5A and 5B, most of the nuclei from normal liver were strongly positive for HNF-4 α by immunostaining. Nuclei that do not contain HNF-4 α are from nonparenchymal cells such as endothelial or Kupffer cells. Treatment with LPS resulted in a marked reduction in the percentage of nuclei that were positive for HNF-4 α , and a reduction in the signal in the few nuclei that contained it. Light microscopy demonstrated that cells with the histologic characteristics of hepatocytes were still present after LPS (data not shown). Because no signal was detected in the cytoplasm, we conclude that LPS results in a decrease in HNF-4 α protein levels rather than causing a redistribution of the protein.

The Decrease in HNF-4 α After Administration of LPS Is Functionally Important. The magnitude of the decrease in HNF-4 α levels that occurs after LPS, and the known importance of this transcription factor in the maintenance of liver-specific functions, led us to predict that LPS would adversely affect the expression of HNF-4 α -dependent genes. We therefore tested the effect of LPS on mRNA levels for HNF-1 α , because the rat HNF-1 α promoter contains an HNF-4 α -specific DR-1 site,³⁵⁻³⁷ and HNF-1 α mRNA levels fall to <10% of normal in HNF-4 α -deficient livers^{20,21} or cell lines.^{25,35,37,38} To evaluate HNF-1 α mRNA levels after LPS treatment, a competitive RT-PCR reaction was used, because other assays were not sufficiently sensitive to yield a quantifiable signal. All liver RNA samples were spiked with 300 fg of a shorter competitor RNA that could be amplified by the same oligonucleotides as the endogenous HNF-1 α mRNA during RT-PCR. The ratio of the longer (mRNA) to the shorter (competitor) DNA product was used to determine the level of HNF-1 α mRNA after comparison with the standard curve generated using *in vitro*-transcribed RNA. As shown in Fig. 6, administration of 15 mg/kg of LPS resulted in HNF-1 α mRNA levels that were $7 \pm 0.7\%$ of that observed in normal liver at 10.5 hours (lanes 23-24; $P = .02$ vs. normal) after correction for the levels of RNA in the sample using a Northern blot for 28S rRNA. The lower dose of LPS resulted in HNF-1 α mRNA levels that were $18 \pm 0.5\%$ of normal at 10.5 hours (lanes 15-16; $P = .03$ vs. normal). The values in rats that received 5 mg/kg of LPS were significantly higher at 10.5 hours than in rats that received 15 mg/kg of LPS ($P = .007$). These data suggest that the decrease in HNF-4 α protein is functionally important, and that the higher dose of LPS has a more severe effect.

HNF-4 α Decreases in HepG2 Cells in Response to IL-1 β . The above data demonstrated that HNF-4 α protein levels fall dramatically after LPS treatment with no major changes in RNA levels. We therefore tested the hypothesis that targeted protein degradation may be responsible. Because analysis of protein degradation in animals would be difficult, HNF-4 α levels were tested in HepG2 cells after treatment with cytokines that are known to be induced during the acute-phase response. HepG2 cells, which express HNF-4 α and degrade I κ B via the proteasome in response to cytokines,^{39,40} were stimulated with both TNF- α and IL-1 β . Samples collected at various times thereafter were analyzed by immunoblot for HNF-4 α protein levels. In addition to the major HNF-4 α isoform at 54

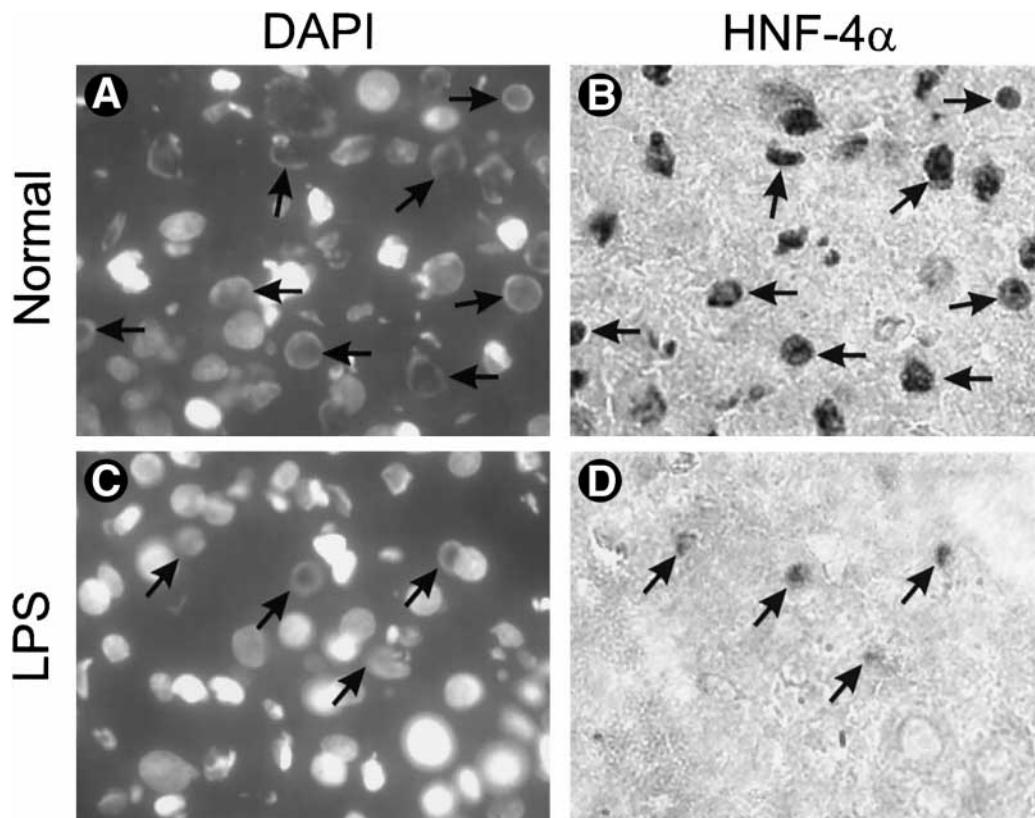


FIG. 5. Immunostaining for HNF-4 α in the liver after LPS treatment. Livers from a normal rat or a rat that was injected with 5 mg/kg of LPS at 4.5 hours before harvest were sectioned and stained with DAPI to identify all nuclei (A and C) or with immunocytochemistry using the C-terminus-specific antibody to identify HNF-4 α (B and D). Arrows identify nuclei from HNF-4 α -positive cells. The colorimetric stain used for the identification of the HNF-4 α -expressing cells reduces the intensity of the DAPI stain in nuclei. The very bright nuclei in (A) are likely from nonparenchymal cells.

kd that comigrates with the protein from normal liver, extracts derived from HepG2 cells had bands at 59 kd and 48 kd that were of variable intensity using the C-terminus-specific anti-HNF-4 α antibody in an immunoblot assay (Fig. 7A). These proteins were not recognized by the N-terminus-specific antibody (not shown), and it is still unclear if they correlate with any of the unusual isoforms that appear after LPS treatment.

At 8 hours after cytokine treatment (lane 9), levels of the 54-kd HNF-4 α protein were 14% of that found in control cells

that received media alone (lanes 2-3), and remained low for up to 24 hours (lanes 10-13). Because Coomassie staining (not shown) demonstrated that similar levels of protein were present in all samples, we conclude that HepG2 cells exhibit a decrease in HNF-4 α levels in response to cytokines. IL-1 β appeared to be the main cytokine responsible, because 10 ng/mL of IL-1 β (Fig. 7B, lanes 5-6; $P = .002$ vs. no cytokine) resulted in a decrease in HNF-4 α levels to 21% of normal, while TNF- α had no significant effect. We next tested if IL-1 β affected HNF-4 α mRNA levels. Although IL-1 β alone resulted

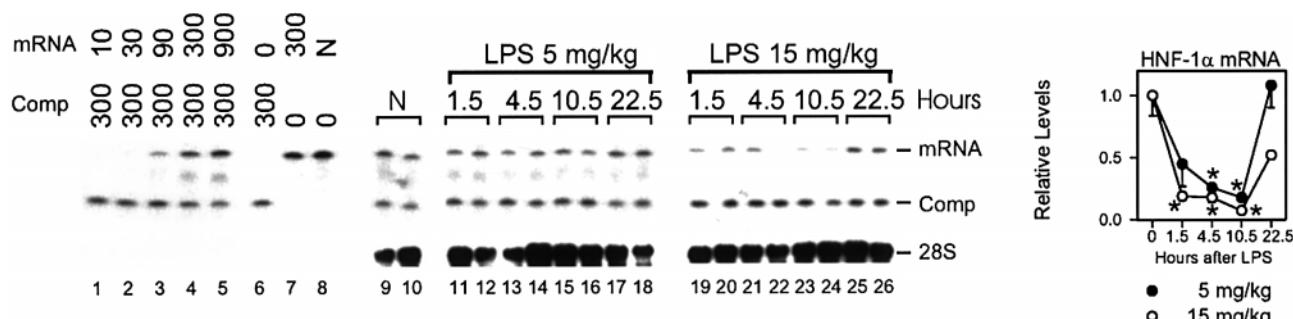


FIG. 6. Analysis of HNF-1 α mRNA levels after LPS. RNA for standards and samples underwent ReT-PCR using HNF-1 α -specific oligonucleotides and radiolabeled dCTP as described in Materials and Methods. Samples for the standard curve are shown in lanes 1 to 5, in which the indicated amount of competitor (Comp) or full-length (mRNA) *in vitro*-transcribed RNA were added at the indicated amounts in femtograms. The sample in lane 6 received only the competitor, that in lane 7 received only the full-length *in vitro*-transcribed RNA, and that in lane 8 received only 2 μ g of DNase-I-treated RNA from a normal liver. All other samples (lanes 9-26) received 2 μ g of DNase I-treated RNA and 300 fg of competitor. Samples were from normal liver (N; lanes 9-10) or from rats that were treated with 5 mg/kg (lanes 11-18) or 15 mg/kg (lanes 19-26) of LPS. The position of the 274-nt PCR product derived from the longer *in vitro*-transcribed RNA or from HNF-1 α mRNA (mRNA), and the 189-nt product derived from the competitor (Comp) are indicated on the right. Duplicates represent samples from 2 different animals that were treated in an identical fashion. For each liver sample, Northern blot analysis was performed with 1 μ g of the same RNA as was used for the ReT-PCR reaction and a 28S rRNA probe to determine the levels of RNA in each sample (28S). The amount of HNF-1 α mRNA relative to that present in normal liver was determined after normalization for the amounts of 28S rRNA in each sample. Values were compared with those found in normal liver by the Student *t* test. * P between 0.05 and .005.

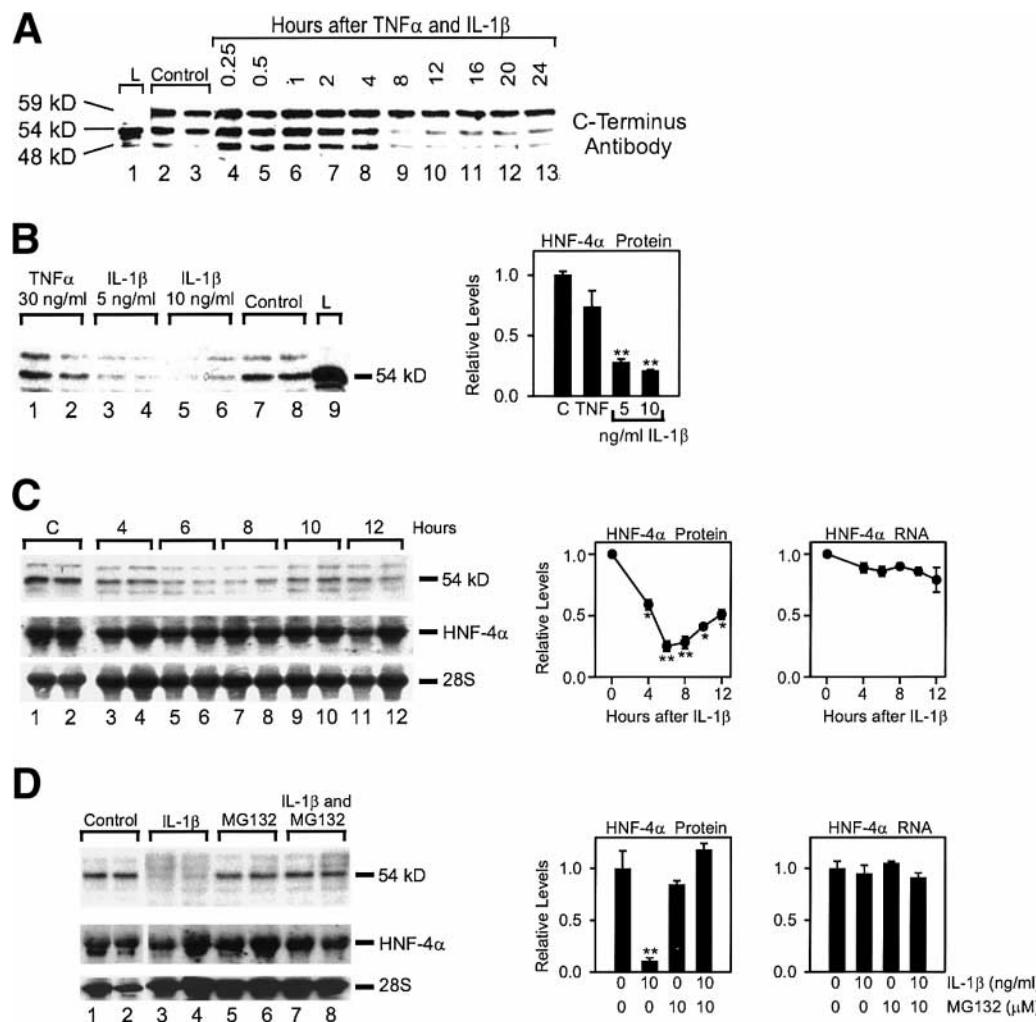


FIG. 7. Evaluation of HNF-4 α protein and mRNA levels in HepG2 cells after cytokine treatment. (A) Time course of HNF-4 α protein levels after treatment with TNF- α and IL-1 β . HepG2 cells were treated with medium without cytokines (control, lanes 2-3) or with 30 ng/mL of TNF- α and 10 ng/mL of IL-1 β (lanes 4-13). Cell extracts were prepared at the indicated number of hours after adding cytokines, and immunoblot analysis was performed using the C-terminus-specific anti-HNF-4 α antibody. The position of the 54-kd HNF-4 α protein is indicated. The sample from normal liver (lane 1) used 20 μ g of nuclear extract protein and a shorter exposure time. (B) Effect of different cytokines on HNF-4 α levels. HepG2 cells were treated with 30 ng/mL of TNF- α alone (lanes 1-2), or with 5 ng/mL (lanes 3-4) or 10 ng/mL (lanes 5-6) of IL-1 β alone, and HNF-4 α levels were determined by immunoblot of extracts that were prepared 8 hours later using the C-terminus-specific antibody. Lane 9 shows an equal amount of nuclear extract protein from normal liver. Levels were normalized to that present in control cells that did not receive any cytokines (lanes 7-8), and statistical comparisons were between experimental samples and these controls using the Student *t* test. ***P* between .005 and .0005. (C) Time course of HNF-4 α protein and mRNA levels in response to IL-1 β . HepG2 cells were treated with normal medium without any cytokine (control, lanes 1-2) or with 10 ng/mL of IL-1 β for the indicated time in hours (lanes 3-12). For some samples, cellular extracts were tested for HNF-4 α protein levels by performing immunoblot with the C-terminus-specific antibody (54 kd). For other similarly treated samples, total RNA was analyzed by Northern blot analysis for HNF-4 α mRNA levels (HNF-4 α), then the blot was stripped and reprobed for 28S rRNA levels (28S) to document that equal amounts of mRNA were loaded. Levels of the HNF-4 α 54-kd protein and the HNF-4 α mRNA (after normalization for 28S rRNA levels) relative to that present in untreated HepG2 cells are shown on the right. (D) Effect of a proteasome inhibitor on HNF-4 α protein and mRNA levels in response to IL-1 β . HepG2 cells were treated with control medium alone (lanes 1-2), with 10 ng/mL of IL-1 β alone (lanes 3-4), with 10 μ M of the proteasome inhibitor, MG132, alone (lanes 5-6), or with IL-1 β and MG132 (lanes 7-8). Cellular extracts were harvested at 8 hours after cytokine treatment, and immunoblot for HNF-4 α protein (54 kd) was performed as noted in (A). Northern blot was performed on total RNA that was isolated from similarly treated cells using an HNF-4 α mRNA probe (HNF-4 α) and then stripped and hybridized with a 28S rRNA probe (28S) for normalization purposes. Values relative to those present in untreated HepG2 cells are shown on the right, and statistical comparisons were between untreated (control) and treated cells.

in a decrease in HNF-4 α protein levels at 4 to 12 hours, there was no effect on HNF-4 α mRNA levels at these times (Fig. 7C). This suggests that the effect of IL-1 β on HNF-4 α protein levels is not caused by alterations in the transcription rate or mRNA stability.

Because cytokines can induce targeted degradation of other proteins via the proteasome, we next tested if a proteasome inhibitor could prevent the decrease in HNF-4 α protein. HepG2 cells were stimulated with IL-1 β , and received either no drug, or received the proteasome inhibitor, MG132, before

the addition of cytokine, as shown in Fig. 7D. Immunoblot of cell extracts obtained 8 hours later demonstrated that treatment with MG132 alone had no effect on HNF-4 α protein levels (54 kd; lanes 5-6) as compared with cells that received no treatment (lanes 1-2). However, HNF-4 α protein levels were 10-fold higher in cells that received MG132 in addition to IL-1 β (lanes 7-8) than in cells that received IL-1 β alone (lanes 3-4; *P* = .004). Northern blot analysis demonstrated that the increase in HNF-4 α protein levels in response to MG132 was not associated with a change in HNF-4 α mRNA

levels. This suggests that proteasome-mediated degradation played a major role in the decrease in HNF-4 α protein levels.

DISCUSSION

The Normal HNF-4 α Isoforms Appear to be Degraded Via the Proteasome in the Liver in Response to LPS. This study demonstrates that LPS treatment results in a marked (>94%) decrease in the major isoforms of HNF-4 α in the nucleus of the liver in rats (Fig. 2). The decrease was not the result of a decline in mRNA levels, as occurs for some other nuclear receptors during the acute-phase response,⁴¹ because HNF-4 α RNA was maintained at normal levels (Fig. 4). In addition, there were no major changes in the normal splicing patterns, because RT-PCR using primers from exons 1 and 3, exons 8 and 9, and exons 8 and 10 demonstrated that the major mRNAs were present at normal or near-normal levels (data not shown). The decrease in HNF-4 α protein levels in nuclear extracts was also not caused by the redistribution of HNF-4 α that occurs in some circumstances,³⁴ because immunocytochemistry failed to demonstrate HNF-4 α in the cytoplasm (Fig. 5).

We favor the hypothesis that targeted degradation of HNF-4 α occurred in the liver, and that this was in large part caused by the proteasome. IL-1 β is a cytokine whose levels increase in response to LPS and other inducers of the acute-phase response.¹ Because this study demonstrated that the administration of IL-1 β resulted in a marked decrease in HNF-4 α protein levels in HepG2 cells without affecting mRNA levels, and a proteasome inhibitor prevented this decline in protein levels (Fig. 7), it is likely that this cytokine contributed to the decrease in HNF-4 α in rats by activating proteasome-mediated degradation in the liver in response to LPS.

Several other nuclear receptors are degraded in a ubiquitin-dependent and/or proteasome-dependent fashion. These include the retinoic acid receptor γ ,⁴² retinoid X receptor α ,⁴² vitamin D receptor,^{43,44} the estrogen receptor,⁴⁵ the thyroid hormone receptor,⁴⁶ and PPAR γ .⁴⁷ Although a PEST sequence^{48,49} is a commonly used signal for targeted degradation, a putative PEST signal played no role in UV light-activated degradation of RAR γ or RXR α .⁴² However, it remains possible that a PEST signal might play a role in targeted degradation of HNF-4 α , because the program PESTfind (<http://www.at.embnet.org/embnet/tools/bio/PESTfind/>) demonstrated that the major isoforms of HNF-4 α contain excellent potential PEST sequences near the C-terminus (aa 412-437 for HNF-4 α 2 [PRPRGQAATPETPQPSPSGSGSESY] and aa 402 to 427 of HNF-4 α 1 [HLSNGQMCTPQPSPSGSGSESY]) that might confer protein instability. Additional studies with deletions of a tagged HNF-4 α gene in HepG2 cells should prove invaluable in mapping the elements responsible for this targeted degradation.

Although levels of the major HNF-4 α isoforms were markedly reduced, there was a 50-kd protein that remained after LPS treatment, and was recognized by two different HNF-4 α -specific antibodies. This protein exhibited a binding specificity that was similar to HNF-4 α , but it clearly did not contain either the N- or the C-terminus of the major isoforms. We favor the hypothesis that this is an unusual HNF-4 α isoform. Indeed, we have identified an mRNA in the liver that encodes the rat HNF-4 α 3, which is formed when the exon 8-to-exon 9 splice does not occur (data not shown). This mRNA would produce a protein that does not contain the C-terminus, and

would lack the putative PEST signal in exons 9 and 10. It should be transcriptionally active, because it still contains the LLQEMLL motif in AF-2 that interacts with coactivators, but no longer contains the inhibitory sequences in domain F.⁵⁰

However, we have not yet identified an mRNA with an alternative 5' end that could explain how a protein without the normal N-terminus could be generated. Specifically, there was no evidence by RT-PCR for the HNF-4 α 7 mRNA (data not shown), which contains an alternative first exon instead of exon 1A, or for the HNF-4 α 4 mRNA (data not shown), which contains additional sequences between exon 1A and exon 2, and could generate an appropriately sized protein without the normal N terminus after proteolytic cleavage. Studies to look for an unusual HNF-4 α isoform, and to determine its transcriptional activity, are in progress. An alternative explanation for the 50-kd protein that binds to a DR-1 site reported to be specific for HNF-4 α is that it represents HNF-4 γ , which is highly conserved in some regions with HNF-4 α .^{51,52} However, HNF-4 γ mRNA levels are very low in the normal rat liver and are not increased in response to LPS (data not shown). In addition, HNF-4 γ protein was not detected on immunoblot, and an anti-HNF-4 γ antibody failed to supershift any of the complexes from livers of LPS-treated rats (data not shown). These data make it unlikely that the 50-kd protein is HNF-4 γ .

Implications for the Treatment of Liver Failure in the Acute-Phase Response. The decrease in HNF-4 α in response to LPS resulted in a decrease in HNF-1 α mRNA, whose expression is highly dependent on HNF-4 α (Fig. 6). Our finding that HNF-1 α mRNA levels are reduced in response to LPS differ from those of Trauner et al.,⁸ who found that LPS resulted in a decrease in HNF-1 α DNA binding activity without affecting its mRNA levels. This discrepancy may be a result of the fact that they used a 5-fold-lower dose of LPS, 1 mg/kg, in their study. We also observed a decrease in HNF-1 α DNA binding activity in response to LPS at both doses (data not shown), which is probably the result of both transcriptional and posttranscriptional mechanisms. In addition to reducing the expression of liver-specific genes in the existing cells, the decrease in HNF-4 α might contribute to cytokine-induced apoptosis of hepatocytes, because HNF-4 α can protect hepatoma cells from undergoing TNF- α -induced apoptosis.⁵³ Thus, the decrease in the major isoforms of HNF-4 α observed in this study is very likely to be of functional significance.

The decrease in HNF-4 α protein levels and expression of HNF-1 α mRNA was more severe for animals that received the higher dose of LPS than for those that received the lower dose. This is consistent with the fact that a higher dose of LPS results in more severe clinical symptoms.⁵⁴ Because both doses resulted in a similar decrease in the 52- to 54-kd isoforms of HNF-4 α , but the lower dose resulted in higher levels of the 50-kd protein, we hypothesize that the 50-kd protein can partially compensate for the decrease in the normal isoforms. Further studies to define the N-terminus of this protein, and to understand what causes its increase in response to LPS, are in progress. It is also possible that a decrease in HNF-4 α could contribute to toxicity in the kidney and intestines in response to LPS. Studies are in progress to determine whether LPS affects HNF-4 α levels in these organs as well.

Finally, it is possible that a decrease in HNF-4 α protein levels could occur in other models of liver insufficiency or failure. Indeed, we have evidence that a 90% partial hepatectomy results in a similar decrease in HNF-4 α levels (Wang B,

Cai S-R, Ponder KP, February 2001, unpublished data), and hypothesize that this is a major factor in the liver insufficiency that ensues.⁵⁵ We plan to test the levels of HNF-4 α in other models of liver insufficiency as well. The finding that the decrease in HNF-4 α appears to involve IL-1 β -induced proteasome-mediated degradation might have implications for the ability to treat patients with liver insufficiency during the acute-phase response. IL-1 receptor antagonist can inhibit the effect of IL-1 β ,⁵⁶ and has been used with some success to treat patients with rheumatoid arthritis.⁵⁷ Thus, if IL-1 β is the major cytokine responsible for the decrease in HNF-4 α levels *in vivo*, it might be possible to prevent the decrease in HNF-4 α in patients with acute-phase response-induced liver insufficiency by administration of IL-1 receptor antagonist. Proteasome inhibitors have been used to inhibit degradation of proteins in animals,^{58,59} and could similarly result in an increase in HNF-4 α levels by blocking its degradation. Future studies will evaluate the ability of these agents to prevent LPS-induced liver insufficiency.

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