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Article

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TNF- α downregulates murine hepatic growth hormone receptor expression by inhibiting Sp1 and Sp3 binding

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Children with chronic inflammatory diseases experience growth failure and wasting. This may be due to growth hormone resistance caused by cytokine-induced suppression of growth hormone receptor (GHR) gene expression. However, the factors governing inflammatory regulation of GHR are not known. We have reported that Sp1 and Sp3 regulate hepatic GHR expression. We hypothesized that TNF- α suppresses GHR expression by inhibiting Sp1/Sp3 transactivators. LPS administration significantly reduced murine hepatic GHR expression, as well as Sp1 and Sp3 binding to *GHR* promoter *cis* elements. TNF- α was integral to this response, as LPS did not affect hepatic Sp1/Sp3 binding or GHR expression in TNF receptor 1-deficient mice. TNF- α treatment of BNL CL2 mouse liver cells reduced Sp1 and Sp3 binding to a *GHR* promoter *cis* element and downregulated activity of a *GHR* promoter-driven luciferase reporter. Combined mutations within adjacent Sp elements eliminated *GHR* promoter suppression by TNF- α without affecting overall nuclear levels of Sp1 or Sp3 proteins. These studies demonstrate that murine GHR transcription is downregulated by LPS, primarily via TNF- α -dependent signaling. Evidence suggests that inhibition of Sp transactivator binding is involved. Further investigation of these mechanisms may identify novel strategies for preventing inflammatory suppression of growth.

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Introduction

Many children with chronic diseases, such as inflammatory bowel disease (IBD) and chronic liver disease, experience poor linear growth and muscle wasting (1, 2). In these diseases, inadequate nutrition and chronic inflammation combine to create a state of acquired growth hormone resistance (3). This resistance to GH may be due to transcriptional downregulation of the growth hormone receptor (GHR) gene and concomitant postreceptor defects in GHR signaling and IGF-1 synthesis (4). GHR plays a critical role in postnatal growth; systemic reduction in GHR levels results in severely stunted growth (5). Moreover, hepatic GHR expression may be essential for normal growth, as evidenced by the miniature *Bos indicus* cattle, which exhibit low levels of hepatic GHR gene expression but normal levels in extrahepatic tissues (6). The mechanisms by which inflammatory cytokines may suppress GHR gene expression in chronic diseases, however, are poorly understood.

A broader appreciation of the role of cytokines in the metabolic complications of a number of chronic diseases has begun to emerge. As a key target of systemic inflammatory mediators, the liver controls acute-phase delivery of metabolic substrates and regulatory proteins to the body, including IGF-1. Evaluation of TNF

receptor and IL-6- and IL-1 β -deficient mice has demonstrated that there is significant redundancy in terms of cytokine regulation of hepatic genes (7). Recent studies have begun to elucidate the manner by which specific cytokines suppress the hepatic GHR gene. Constitutive overexpression of TNF- α in transgenic mice reduces circulating levels of IGF-1, presumably via decreased expression of the GHR gene, and is associated with stunted growth (8). TNF- α and IL-1 β directly inhibit growth hormone-induced hepatic GHR expression and IGF-1 synthesis in primary rat hepatocytes (9). However, the associated mechanism by which this inhibition occurs has not been defined.

On the assumption that this regulation could be at the level of gene transcription, we have begun to analyze regulatory elements that mediate expression of the murine GHR gene (10). Two different 5' untranslated exons, L1 and L2, have been described elsewhere (11). The expression of the associated L1 and L2 transcripts is regulated by distinct promoters (11). The L2 transcript is the predominant GHR transcript in both hepatic and extrahepatic tissues in the nonpregnant state (12). Our initial analysis of the L2 promoter has identified two adjacent Sp family regulatory elements (GC boxes) (10). Several different types of cellular

stimuli, including cytokines, have been shown to affect Sp1 DNA binding (13). In many cases, this has been associated with changes in Sp1 phosphorylation (14). Additional GC box binding proteins, such as BTEB and G10BP-1, have also been identified that could affect overall transcription from Sp elements in an inducible fashion (15). Moreover, specific inducible Sp proteases have been reported that can selectively reduce overall nuclear levels of Sp1 (16). Whether any of these mechanisms were playing a role in altering Sp factor DNA binding and associated GHR promoter activity in response to cytokines was not known.

We hypothesized that inflammatory suppression of the GHR gene would occur via cytokine-induced downregulation of key Sp transactivator proteins. In this study, we demonstrate that the murine GHR gene is downregulated by LPS and TNF- α and that this appears to occur via inhibition of Sp transactivator binding to the L2 promoter of the GHR gene.

Methods

Oligonucleotides. The following synthetic oligonucleotides were used in these experiments (residues altered in the mutant oligonucleotide are indicated in lowercase type): L2-A: murine GHR: ⁻⁶⁵TTTACCCCGC-CCCCTTCTC⁻⁴⁵; L2-B: murine GHR: ⁻⁹⁸CCTCTCCCCTCCCCTCCCCTCCTCCCTCCCAG⁻⁶⁶; L2-A-m: ⁻⁷⁸CCTCCTTCCCAGTTTACCTaaagCCTTCTCCTCCCCAAGCC⁻³⁴; L2-B-m: ⁻¹⁰⁸CCCCTCTTCTCCTCCTCCagaagagaaCTCCTCCCTCCCAG⁻⁶⁶; and HNF1: murine albumin: ⁻⁷⁰AGTATGATCGAGATCTTACAG⁻⁵⁰.

When necessary double-stranded oligonucleotides were generated by annealing of synthetic oligonucleotides with the respective complementary sequences.

The following primers and probes were used for the TaqMan RT-PCR assay: GHR: forward: GGATCTTTGTCAGGTCTTCTTAACCT; reverse: CAAGAGTAGCTGGTGTAGCCTCACT; probe: TGGCAGTCACCAGCAGCAGCATTTT. L2: forward: GTCCACGCGCCTGAG; reverse: TCGCCTCGGGAGACAGAAC; probe: CAGCCCCAAGCGGACACGA. GAPDH: proprietary (Perkin-Elmer Biosystems, Foster City, California, USA).

Materials and plasmids. Cell culture media and Lipofectamine were purchased from Life Technologies Inc. (Rockville, Maryland, USA). BNL CL2 mouse liver cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Six-week-old male C57BL/6J wild-type and TNF receptor 1-knockout mice (Tnfrsfla, catalog no. 002818) were obtained from The Jackson Laboratory Bar Harbor, Maine, USA). Murine cytokines were obtained from R&D Systems Inc. (Minneapolis, Minnesota, USA). Luciferase assay reagents were obtained from Promega Corp. (Madison, Wisconsin, USA). LPS, *Escherichia coli* 055:B5 was obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Sp1 rabbit polyclonal antibody was obtained from Sigma Chemical Co.; IgG and Sp3 rabbit polyclonal antibodies and immunoprecipitation agarose beads were obtained from Santa Cruz Biotechnology Inc. (Santa

Cruz, California, USA). The Western Blot Chemiluminescence Reagent Plus kit was obtained from NEN Life Science Products Inc. (Boston, Massachusetts, USA).

LPS treatment. Endotoxemia was induced by a single intraperitoneal injection (1 μ g/g body weight). At 12 hours after treatment, livers were harvested for preparation of nuclear proteins and RNA. The study protocol was approved by the Yale Animal Care and Use Committee.

Northern blot and RT-PCR analysis of alterations in GHR gene expression. Total RNA was isolated from control and treated mouse liver using TRIzol (Life Technologies Inc.). Northern blot analysis for total GHR was carried out by standard techniques. The ³²P-labeled probe for exon 4 of the murine GHR (12) was generated by random prime labeling (Boehringer Mannheim, Indianapolis, Indiana, USA). After autoradiography, the probe was stripped from the membrane according to methods recommended by the manufacturer and was rehybridized with an oligonucleotide probe for 18 S RNA (17). Total and L2 transcripts of the murine GHR gene were also quantified by the fluorescent 5'-nuclease (TaqMan) assay using the ABI Prism 7700 Sequence Detection System (PE Biosystems) (18, 19). The GHR and GAPDH probes were labeled with fluorescent reporter dyes VIC and FAM, respectively. The relative efficiencies of the GHR primers/probe sets and the GAPDH primer/probe pair were tested by subjecting serial dilutions of a single RNA sample. The plot of log input versus ΔC_T was <0.1 , which satisfies the previously established criterion for equivalence of efficiency of amplification. After confirming that the efficiency of amplification of the GHR transcripts and the GAPDH transcripts were approximately equal, the limiting primer concentrations were defined according to the previously established protocols. These experiments established that the desired primer concentrations of both the forward and reverse primers that result in a reduction in ΔR_n but no effect on C_T were 60 and 200 nM for the GAPDH and the GHR transcripts, respectively. Following these standardization and validation assays, the amount of GHR transcripts relative to the GAPDH transcript was determined by multiplex PCR using the comparative C_T method. Briefly, 2 ng aliquots of total RNA were analyzed using the One-Tube RT-PCR protocol (PE Biosystems). After RT at 48°C for 30 minutes, the samples were subjected to PCR analysis using cycling parameters: 95°C \times 10 minutes; 95°C \times 15 sec \rightarrow 60°C \times 1 minutes for 40 cycles. Each sample was analyzed in triplicate in individual assays performed on two or more occasions.

Cell culture, plasmid constructs and stable and transient transfections. BNL CL2 mouse liver cells were maintained in DMEM supplemented with 10% FBS and penicillin-streptomycin and grown to 50% confluence before transfection or nuclear protein preparation. A plasmid containing the murine L2 GHR promoter sequence from -2kb/+110 (p-L2luc) has been reported previously (10). Internal mutations of two adjacent Sp elements, L2-A (nucleotides [nt] -65/-45) and L2-B (nt -98/-66) were

introduced using oligonucleotides L2-A-m and L2-B-m in the QuikChange (Stratagene, La Jolla, California, USA) protocol, to yield p-L2m1luc (mutation in L2A only), p-L2m3luc (mutation in L2B only), and p-L2(m1+m3)luc (mutation in L2A and L2B). An RSV-driven β -galactosidase expression plasmid, p-RSV β Gal, was used as the internal control for transfection efficiency (20). For stable transfection, BNL CL.2 cells were seeded in 150-mm-diameter plates at a density of 1×10^6 to 2×10^6 cells per plate. After 24 hours, using the Lipofectamine protocol the cells were transfected with p-L2luc and pCR3.1-Uni (Invitrogen Corp., San Diego, California, USA), which encoded the gene conferring resistance to G418. After 2 days, cells were split at a ratio of 1:10–20, and stable transfectants were selected in G418 (1,200 mg/ml; Life Technologies Inc.). BNL CL.2 cells were also transiently transfected using the Lipofectamine technique, as reported previously (10).

Cytokine treatments. After transfection, cytokines were added to serum-free media for treatment of cells for 4–24 hours. Dose responsiveness of 4- to 24-hour treatments with TNF- α , IL-1 β , or IL-6 (1–100 ng/ml) was determined. Luciferase and β -galactosidase activities were assayed according to manufacturer's directions (Promega Corp.).

Preparation of nuclear proteins, EMSA, and Western blot analysis. Nuclear proteins were prepared from mouse liver or BNL CL.2 cells as described previously (20, 21). Oligonucleotide probes were end labeled and EMSA was performed as described, using 5 or 10 μ g of nuclear extracts per incubation (20, 21). In supershift assays, 2 μ g of polyclonal antibody was added to the gelshift reaction for 1 hour after incubation of the probe and nuclear proteins for 20 minutes on ice. The total amount of antibody used was maintained at 2 μ g in all supershift reactions. In other experiments, Sp1 or Sp3 was immunoprecipitated from nuclear extracts before EMSA or immunoblotting using agarose beads according to the manufacturer's recommendations (Santa Cruz Biotechnology Inc.). IgG was used as a control for these experiments. Nuclear proteins (20 μ g) were also loaded onto 7.5% SDS-polyacrylamide gels and subjected to electrophoresis and electrotransfer onto nitrocellulose membranes. Uniformity of protein loading and transfer was assessed using Ponceau staining. Nitrocellulose membranes were blocked overnight at 4°C in 20 mM TrisHCl, 150 mM NaCl, 5% nonfat dry milk, 0.1% Tween-20 (TS complete). Blots were incubated at room temperature for 2 hours with Sp1 (1:1,000) or Sp3 (1:2,000) rabbit polyclonal antibodies in TS complete. Blots were washed and then incubated with anti-rabbit HRP conjugate antibody (1:5,000; Santa Cruz Biotechnologies) for 2 hours at room temperature. Immune complexes were detected using the NEN Chemiluminescence Plus Western blotting kit.

Statistical analysis. Data were expressed as the mean \pm SD or the mean \pm SE of experiments with at least three independent treatments per group. Differences among experimental groups were analyzed by ANOVA.

Results

Endotoxin-induced TNF- α suppresses murine hepatic GHR gene expression. LPS administration is well established as a method for determining effects of inflammatory cytokines such as TNF- α upon hepatic gene expression (7, 22). Moreover, it has recently been reported that LPS treatment reduced hepatic GHR and IGF-1 expression in rats, although the underlying molecular mechanism was not determined (23). Wild-type (WT) and TNF receptor 1-deficient C57BL/6 male mice were treated with LPS, and livers were harvested 12 hours later. Effects of LPS administration upon hepatic GHR expression were determined by Northern blot (Figure 1a) and real-time quantitative RT-PCR (Figure 1b). RNA levels for both total GHR transcripts and GH binding protein (GHBP), which is encoded by a truncated GHR message, were reduced by LPS treatment of WT mice (Figure 1a). In WT mice, total hepatic GHR expression was reduced by approximately 70% by LPS, whereas expression of the L2 transcript was reduced by approximately 40% (Figure 1b). We have

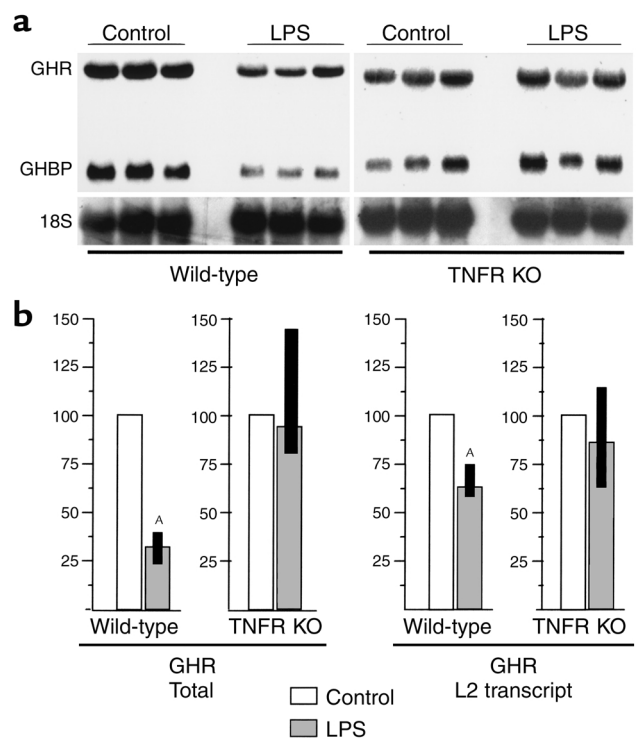
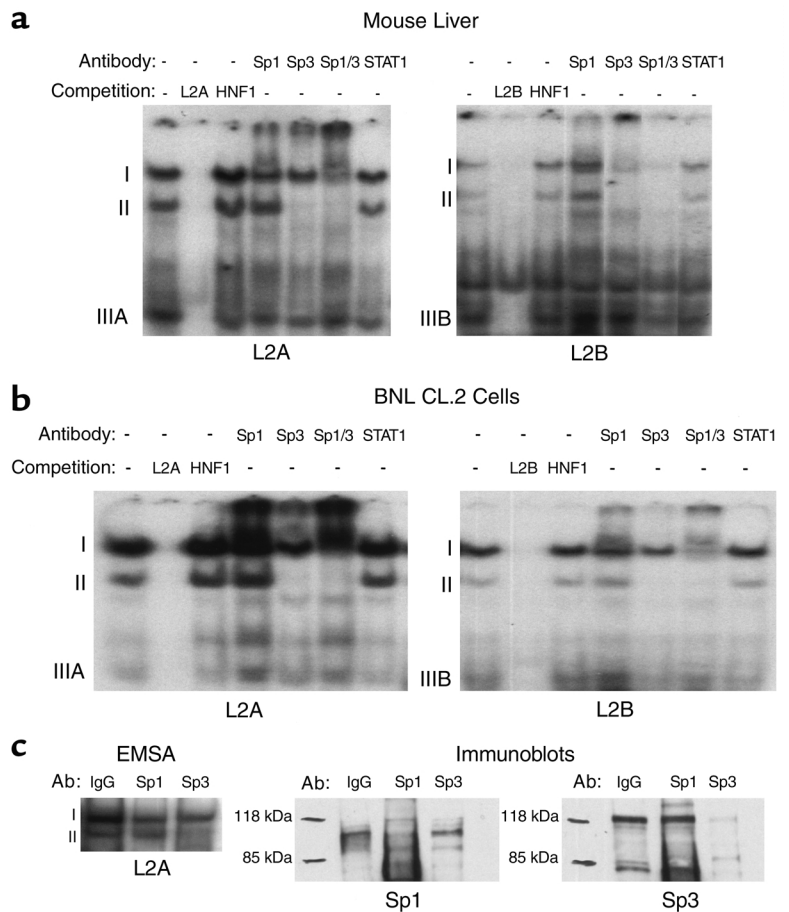


Figure 1

Endotoxin-induced TNF- α suppresses hepatic GHR gene expression. WT and TNF receptor 1-deficient mice were treated with LPS and total liver RNA was harvested after 12 hours. (a) Northern blots for total hepatic GHR and GHBP transcripts and (b) quantitative TaqMan RT-PCR for total and L2 hepatic GHR transcripts and a GAPDH internal control were performed. The relative amounts of each transcript in LPS-treated mice were calculated as a percentage of the corresponding value in control mice. The results are depicted as mean \pm range (filled bar). The range is determined by evaluating the expression: $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s is the SD of the $\Delta\Delta C_T$ value (18, 19). $n = 3$. $^{A}P < 0.01$ (ANOVA) versus control. TNFR KO, TNF receptor 1 knockout mouse.

Figure 2

Sp1 and Sp3 bind the L2 GHR promoter via adjacent response elements. EMSA was performed using nuclear proteins from (a) adult mouse liver or (b and c) BNL CL.2 mouse liver cells and radiolabeled L2A or L2B elements. Supershift (a and b) or immunodepletion (c) with Sp1, Sp3, IgG, or STAT1 antibodies was performed. (c) EMSA for nuclear extracts that were immunodepleted using Sp1, Sp3, or control IgG antibodies before EMSA with the L2A element is shown on the left. Immunoblots confirming specific Sp1 or Sp3 depletion in these extracts are shown on the right. Ab: antibody used for immunodepletion; I: L2A or L2B complex containing both Sp1 and Sp3 proteins; II: L2A or L2B complex containing only Sp3; IIIA or IIIB: faster migrating complexes – L2A or L2B, respectively – which do not contain Sp1 or Sp3; HNF1: HNF1 consensus oligonucleotide.



determined in this and in previous work, using either real-time RT-PCR or RNase protection, that the L2 fraction in control mice represents 40–60% of total hepatic GHR transcripts (10). Thus, both the L2 GHR transcript and an additional, as yet unidentified GHR transcript, were suppressed by LPS. We then tested the specific role of TNF- α signaling in mediating LPS-induced suppression of hepatic GHR gene expression, via administration of LPS to TNF receptor 1-deficient mice. Importantly, LPS treatment did not change either total or L2-associated hepatic GHR gene expression in TNF receptor 1-deficient mice (Figure 1), indicating that TNF- α signaling was required for LPS-induced downregulation of the GHR gene in vivo.

Tandem Sp response elements regulate the GHR promoter. We have reported that basal expression of the L2 GHR transcript is primarily dependent on an Sp response element located near the start site of transcription (L2A, nt -65/-45) (10). We have now identified a second, adjacent Sp element (L2B, nt -98/-66), which also contributes to hepatic expression of the L2 GHR transcript (see Figures 2 and 4). We have previously determined that either Sp1 or Sp3 can transactivate the L2 GHR promoter via the L2A element (10). Internal mutations of the L2A or L2B GC boxes either separately or in combination significantly reduced L2 promoter basal activity in BNL CL.2 mouse liver cells (see Figure 4). EMSA was performed using nuclear proteins from adult mouse liver and BNL CL.2 mouse liver cells to determine the binding specificity of the L2B element relative to the L2A element. The shifted L2A or L2B complex was composed of three specific complexes, I, II, and IIIA

or IIIB. With the exception of the most rapidly migrating complex (IIIA versus IIIB), the gel mobility and Sp1/Sp3 binding were quite similar for these *cis* elements (Figure 2). Coincubation with Sp1 or Sp3 antibodies suggested that both Sp1 and Sp3 comprise complex I, while complex II is comprised of only Sp3. As previously reported for the L2A element, these experiments suggested that the L2B element also binds more Sp3 than Sp1 in murine liver nuclear extracts (10). The identity of the proteins bound in complexes IIIA and IIIB was not determined, as neither the Sp1 nor Sp3 antibodies affected these complexes. It should be noted that a modest signal remained for complex I following co-incubation with both Sp1 and Sp3 antibodies; therefore, an additional GC box binding protein may also comprise this complex.

To confirm that Sp1 and Sp3 comprise complex I of the GHR gene promoter Sp *cis* elements, nuclear extracts from BNL CL.2 cells were also selectively immunodepleted of either Sp1 or Sp3 prior to EMSA. The signal intensity for complex I was reduced by either Sp1 or Sp3 immunodepletion, confirming that both proteins comprise this complex (Figure 2c, left panel). Immunoblots using Sp1 and Sp3 antibodies confirmed that the nuclear extracts had been depleted of Sp1 or Sp3 (Figure 2c, right panel).

Intact TNF- α signaling is required for LPS-induced Sp factor binding downregulation. We performed EMSA using

hepatic nuclear extracts from control and LPS-treated wild type mice to determine whether LPS administration would alter protein/DNA binding to the L2A and L2B elements. As shown in Figure 3a, LPS treatment significantly reduced overall binding of Sp1 and Sp3 to both the L2A and the L2B elements (see complexes I and II). The observed LPS-induced suppression of Sp1 and Sp3 binding to L2 GHR regulatory *cis* elements would likely account for the profound downregulation in gene expression (Figure 1). We also examined the effect of LPS treatment on protein/DNA binding to L2 complex IIIA and IIIB (Figure 3b). LPS treatment led to reduction in binding to both complexes IIIA and IIIB. Investigations are ongoing to determine the identity and functional significance of these DNA binding proteins.

We then determined the effect of LPS treatment upon transcription factor binding to the L2A and L2B elements in TNF receptor 1-deficient mice. As shown in Figure 3c, protein/DNA binding to L2A complexes I and II, as well as IIIA or IIIB, was preserved in LPS-treated TNF receptor 1-deficient mice. This indicated that intact TNF- α signaling was required to mediate LPS-induced reduction in transcription factor binding to the L2A or L2B elements. Preservation of L2A and L2B transcription factor:DNA binding like-

ly accounted for the lack of effect of LPS upon L2 GHR gene expression.

The albumin gene is a prototypical hepatic negative acute-phase gene, which is also suppressed by LPS administration to rodents (24). Coadministration of monoclonal TNF- α antibody does not prevent suppression of albumin gene expression by LPS, indicating that alternative cytokines may be responsible for albumin downregulation in vivo (24). To ensure that inflammatory signals were activated in LPS-treated TNF receptor 1-deficient mice, and to examine the specificity of Sp factor binding preservation, we also determined the effect of LPS treatment upon binding of the HNF1 transcription factor to its *cis* element within the mouse albumin promoter (25). We found that LPS treatment reduced HNF1 binding to the albumin promoter in both WT and TNF receptor 1-deficient mice (Figure 3d). These data indicated that inflammatory signals affecting DNA binding of liver-enriched transcription factors were activated in LPS-treated TNF receptor 1-deficient mice, and that the importance of intact TNF- α signaling in mediating the effects of LPS was transcription factor-specific and thus target gene-specific.

TNF- α or IL-1 β suppress L2 GHR promoter activity via Sp response elements. The results in the WT and TNF receptor 1-deficient mice indicated that TNF- α played a crit-

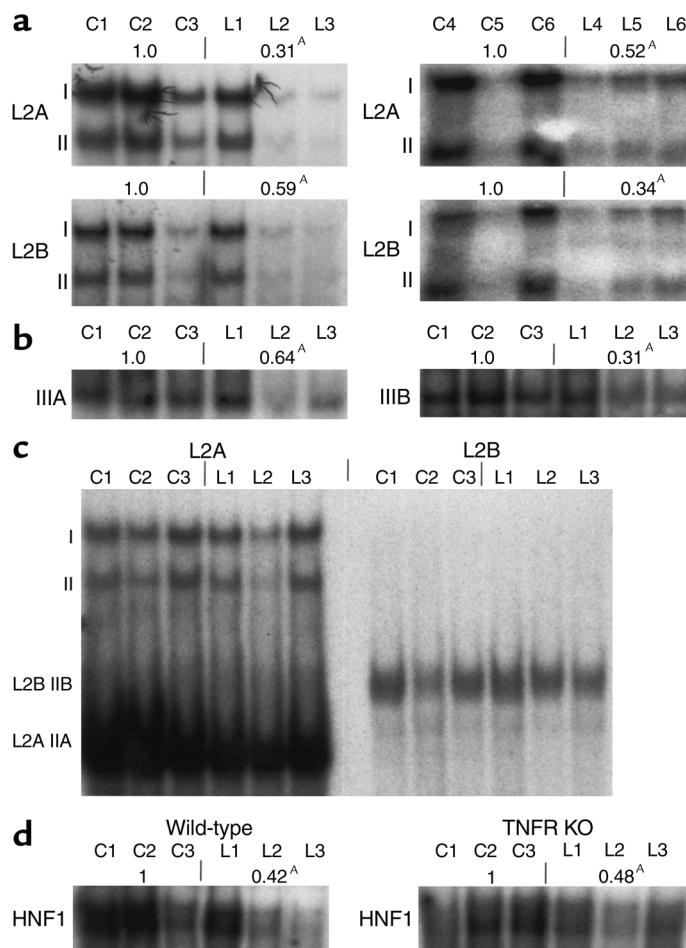
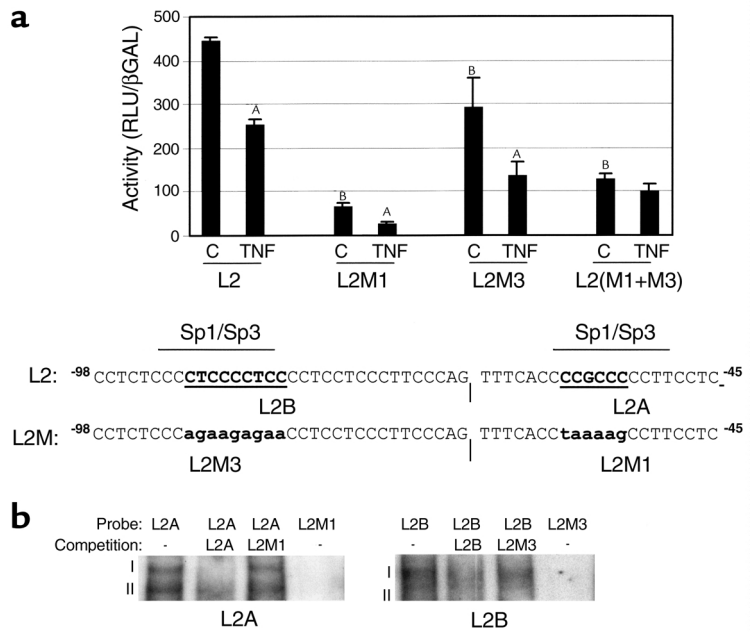


Figure 3

Endotoxin-induced TNF- α downregulates L2 GHR promoter Sp transactivators. WT and TNF receptor 1-deficient mice were treated with LPS, and liver nuclear proteins were isolated after 12 hours. EMSA was performed using L2A, L2B, or HNF1 probes. (a) LPS-induced changes in binding to L2A or L2B complexes I and II in WT mice. (b) LPS-induced changes in binding to L2A or L2B complexes IIIA and IIIB in WT mice. (c) Effects of LPS treatment on binding to L2A or L2B in TNF receptor 1-deficient mice. (d) LPS-induced changes in binding to the HNF1 element in WT and TNF receptor 1-deficient mice. Changes in signal intensity were quantified by densitometry. The mean value for each group of three mice is shown. ^A*P* < 0.05. C, control; TNFR KO, TNF receptor 1 knockout mouse; L, LPS-treated mouse.

Figure 4

TNF- α downregulates GHR L2 promoter activity via the L2A and L2B Sp response elements. (a) WT and mutated GHR L2 promoter luciferase reporter constructs were transiently transfected in mouse liver BNL CL.2 cells and treated with TNF- α (10 ng/ml for 12 hours). Luciferase-specific activity in the cell homogenates was equalized for transfection efficiency monitored by cotransfection of a plasmid expressing β -gal (RLU/ β GAL). Data are expressed as the mean \pm SEM of three independent transfections performed in triplicate. ^A*P* < 0.05 versus control for each plasmid with TNF treatment. ^B*P* < 0.05 versus L2 control plasmid for basal activity. (b) EMSA was performed using mouse liver nuclear proteins and L2A, L2B, L2M1, and L2M3 oligonucleotides.



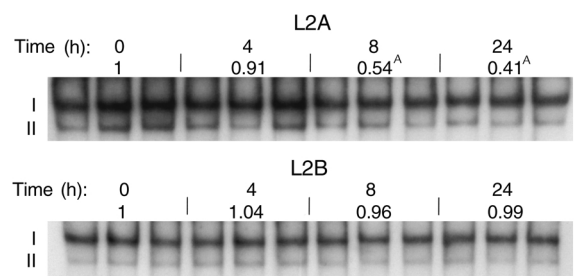
ical role in modulating GHR gene expression in vivo (Figure 1). To determine whether TNF- α played a direct role in regulating L2 GHR gene expression, an approximately 2-kb L2 promoter-driven luciferase reporter construct was stably transfected in BNL CL.2 mouse liver cells, which were then treated with TNF- α , IL-1 β , or IL-6, at doses of 1–100 ng/ml for 6–24 hours. Both TNF- α and IL-1 β downregulated L2 GHR promoter activity by approximately 30%. This effect was maximal by 12 hours after treatment, with a dose of 10 ng/ml (data not shown). By contrast, treatment with IL-6 did not affect L2 GHR promoter activity, indicating that it is unlikely that this cytokine is directly suppressing GHR gene expression in vivo (data not shown).

WT and mutated L2 promoter-driven luciferase reporter constructs were then transiently transfected in BNL CL.2 cells and treated with TNF- α (10 ng/ml for 12 hours). EMSA using competition with an excess of unlabeled L2M1 or L2M3 oligonucleotides or using labeled L2M1 or L2M3 as the probes demonstrated that the M1 and M3 mutations eliminated complex I and II binding in L2A or L2B, respectively (Figure 4b). Individual mutations within the L2A (M1) or L2B (M3) elements significantly reduced basal L2 promoter activity (M1 > M3) but did not affect promoter suppression by TNF- α . However, combined mutation of both the L2A and L2B elements reduced L2 GHR promoter activity by 70%, and abrogated suppression by TNF- α (Figure 4a). Thus, the presence of both adjacent Sp response elements, L2A and L2B, was required for maximal basal promoter activity and promoter suppression by TNF- α .

TNF- α suppresses Sp factor binding to GHR response elements in BNL CL.2 cells. EMSA and Western blot analysis were then performed to examine TNF- α -induced alterations in Sp factor binding to the L2A and L2B *cis* elements. Nuclear proteins were obtained from control and TNF- α -treated BNL CL.2 cells. As shown in

Figure 5, TNF- α treatment significantly suppressed protein/DNA binding in L2A complexes I and II. The L2A suppression was maximal by 8 hours of treatment, preceding the nadir in promoter activity observed at 12 hours. However, protein/DNA binding for the L2B element was not affected. Thus, while these data support the direct role of TNF- α in suppressing L2A complexes I and II, they also indicate that additional TNF- α -dependent pathways affecting protein/DNA binding in L2B complexes I and II are also likely activated in vivo (see Discussion).

TNF- α does not alter overall nuclear levels of the Sp1 or Sp3 proteins. Alterations in DNA-binding activity of Sp1 or Sp3 on EMSA could be due to cytokine-induced changes in overall nuclear levels of these proteins consequent to altered protein production or degradation. To examine this possibility, BNL CL.2 cells were treated with TNF- α (10 ng/ml for 12 hours), and immunoblots were performed using Sp1 or Sp3 antibodies. Total nuclear levels of Sp1 or Sp3 were not

**Figure 5**

TNF- α suppresses transcription factor binding to the GHR L2A and L2B response elements. BNL CL.2 cells were treated with TNF- α (10 ng/ml for 4–24 hours), and nuclear proteins were isolated. EMSA was performed using oligonucleotides corresponding to the L2A and L2B response elements. Changes in signal intensity were quantified by densitometry and are shown. ^A*P* < 0.05.

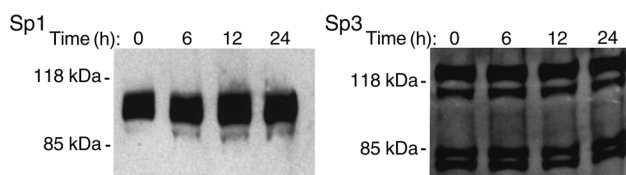


Figure 6
TNF- α does not alter overall nuclear levels of the Sp1 or Sp3 proteins. BNL CL.2 cells were treated with TNF- α (10 ng/ml for 6–24 hours), and nuclear proteins were isolated. Immunoblots were performed using polyclonal Sp1 or Sp3 antibodies as described in Methods. A representative blot is shown; $n = 3$ –5.

affected by TNF- α treatment (Figure 6). Moreover, no clear change in electrophoretic mobility was observed for either the Sp1 or Sp3 bands.

Discussion

Cytokines have been associated with growth hormone resistance, depressed circulating IGF-1 levels, and stunted growth in children with chronic inflammatory diseases (4). Moreover, children with chronic liver diseases and poor growth exhibit decreased hepatic expression of GHR and IGF-1 (2). The importance of GHR for normal postnatal growth has been confirmed in patients with Laron dwarfism, which is caused by mutations of the GHR coding region (5). However, the relative importance of undernutrition versus chronic inflammation in suppressing GHR gene expression and stunting growth has been difficult to determine (1). In this study, we have identified a mechanism for TNF- α -induced suppression of GHR gene expression, via inhibition of Sp transactivator binding to the GHR promoter.

A feature that is common to GHR transcripts from different species is sequence heterogeneity in the 5'-untranslated region (UTR) (12). Two UTRs, termed L1 and L2, have been identified for the murine GH receptor gene. The L2 transcript represents the majority of GHR transcripts expressed in the tissues of non-pregnant mice. In the present study, LPS administration decreased the expression of total GHR transcripts by 70%, whereas the decrease in the L2 transcript was approximately 40%. This differential alteration in the levels of the total and L2 transcript suggests the possibility that LPS administration may also act to decrease GHR expression by decreasing the expression of an as-yet unidentified GHR transcript(s).

IL-6, TNF- α , and IL- β have redundant effects in modulating hepatic gene expression (7). Previous reports have indicated that both TNF- α and IL-1 β can suppress the GHR gene in vitro (9). Whether TNF- α or IL-1 β was predominant in suppressing the GHR gene in vivo was not known. To examine this, we determined the effect of LPS administration to WT versus TNF receptor 1-deficient mice. TNF receptor 1-deficient mice were resistant to LPS-induced suppression of Sp transactivators and associated hepatic GHR gene expression, compared with a 70% reduction in total GHR gene expression in WT mice. This indi-

cated that intact TNF- α signaling was required for LPS-induced GHR gene downregulation. It was possible that this was due to reduced production of a downstream TNF- α -induced cytokine. However, the results in TNF- α -treated BNL CL.2 cells indicated that TNF- α could directly suppress GHR Sp1/Sp3 transactivators and associated GHR gene promoter activity. Therefore, it is likely that TNF- α is also directly mediating LPS-induced GHR gene downregulation in vivo, and potentially suppressing the GHR gene in other inflammatory conditions, such as IBD, in which TNF- α production is increased.

Mutations within the L2A and L2B elements indicated that they function in a cooperative fashion to mediate basal hepatic expression of the GHR gene, with the L2A element accounting for the majority of the promoter activity. This is consistent with prior reports of cooperative regulation of gene promoters by adjacent Sp elements (13). Mutation of both *cis* elements was required to prevent promoter downregulation by TNF- α , despite that fact that, in BNL CL.2 cells, reduced Sp1/Sp3 binding was only observed for the L2A element. This was in contrast to the significant reduction in binding to both *cis* elements observed after LPS administration to mice. This represents a potentially important difference between the mouse and cell culture models and may be due to the effects of alternate downstream cytokines or cytokine signaling pathways induced by LPS in vivo (22). However, previous reports have demonstrated that exogenous factors may influence Sp1 transcriptional function without affecting overall nuclear levels or DNA binding. For example, two adjacent Sp1 elements regulate expression of the HIV LTR promoter. Okadaic acid treatment has been shown to increase Sp1 phosphorylation and induce activation of the HIV promoter domain via these Sp elements, without any demonstrable change in either Sp1 nuclear levels or DNA binding (26). Moreover, it has recently been reported that LPS-dependent induction of the IL-10 promoter in a macrophage cell line also occurs via an Sp1/Sp3 *cis* element, without a change in protein-DNA binding (27). Therefore, it is conceivable that TNF- α treatment of BNL CL.2 cells may also affect the transactivational function of Sp1/Sp3 independent of the overall degree of DNA binding. This could involve a direct effect on phosphorylation or glycosylation of Sp1/Sp3 or an indirect effect involving an Sp1/Sp3 coactivator.

Multiple factors that may contribute to acquired growth hormone resistance, including cytokines and nutrient levels, have been shown to affect Sp1 DNA binding (14, 28). One mechanism to account for changes in Sp1/Sp3 DNA binding would be via alterations in nuclear levels of these proteins. In fact, specific, inducible Sp proteases have been reported that can selectively degrade Sp1. However, immunoblot data (Figure 6) indicated that alterations in Sp1 and Sp3 DNA binding on EMSA were not due to changes

in nuclear levels of these proteins. Therefore, it would appear likely that an alternate mechanism, such as phosphorylation of Sp1/Sp3 or induction of an inhibitory factor, was responsible for the reduced DNA binding and transactivational function (15, 29). Better understanding of these mechanisms of cytokine-induced growth hormone resistance should lead to more specific treatments to improve growth in children with inflammatory diseases.

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