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Interleukin-6 inhibits hepatic growth hormone signaling via upregulation of Cis and Socs-3

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Denson, Lee A., Matthew A. Held, Ram K. Menon, Stuart J. Frank, Albert F. Parlow, and Dodie L. Arnold. Interleukin-6 inhibits hepatic growth hormone signaling via upregulation of Cis and Socs-3. *Am J Physiol Gastrointest Liver Physiol* 284: G646–G654, 2003. First published January 2, 2003; 10.1152/ajpgi.00178.2002.—Cytokines may cause an acquired growth hormone (GH) resistance in patients with inflammatory diseases. Anabolic effects of GH are mediated through activation of STAT5 transcription factors. We have reported that TNF-α suppresses hepatic GH receptor (GHR) gene expression, whereas the cytokine-inducible SH2-containing protein 1 (Cis)/suppressors of cytokine signaling (*Socs*) genes are upregulated by TNF-α and IL-6 and inhibit GH activation of STAT5. However, the relative importance of these mechanisms in inflammatory GH resistance was not known. We hypothesized that IL-6 would prevent GH activation of STAT5 and that this would involve Cis/Socs protein upregulation. GH ± LPS was administered to TNF receptor 1 (TNFR1) or IL-6 null mice and wild-type (WT) controls. STAT5, STAT3, GHR, Socs 1–3, and Cis phosphorylation and abundance were assessed by using immunoblots, EMSA, and/or real time RT-PCR. TNF-α and IL-6 abundance were assessed by using ELISA. GH activated STAT5 in WT and TNFR1 or IL-6 null mice. LPS pretreatment prevented STAT5 activation in WT and TNFR1 null mice; however, STAT5 activation was preserved in IL-6 null mice. GHR abundance did not change with LPS administration. Inhibition of STAT5 activation by LPS was temporally associated with phosphorylation of STAT3 and upregulation of Cis and Socs-3 protein in WT and TNFR1 null mice; STAT3, Cis, and Socs-3 were not induced in IL-6 null mice. IL-6 inhibits hepatic GH signaling by upregulating Cis and Socs-3, which may involve activation of STAT3. Therapies that block IL-6 may enhance GH signaling in inflammatory diseases.

STAT5; STAT3; suppressors of cytokine signaling; endotoxin; cytokines

GROWTH HORMONE (GH) resistance, characterized by normal GH secretion with impaired production of GH target genes including IGF-I, is an important complication of acute and chronic inflammatory diseases (3). Consequences include linear growth failure, skeletal muscle wasting, poor wound healing, and abnormalities in carbohydrate and lipid metabolism (2). Binding of GH to the GH receptor (GHR) activates JAK2 kinase, leading to phosphorylation and nuclear translocation of STAT5 transcription factors and upregulation of anabolic target genes including IGF-I (33). Potential mechanisms of cytokine induced GH resistance include downregulation of the GHR itself, and upregulation of postreceptor inhibitory proteins including members of the recently described suppressors of cytokine signaling (SOCS) family (13, 27).

We have recently reported that TNF-α signaling mediates downregulation of hepatic *Ghr* RNA expression by LPS, via inhibition of Sp1/Sp3 *Ghr* gene transactivators (13). A significant reduction in GHR abundance could then impair GH signaling. Several groups have demonstrated that cytokine-inducible SH2-containing protein 1 (Cis) and Socs 1–3 can inhibit GH signaling by preventing GHR/JAK2-dependent phosphorylation of STAT5b (7, 27, 28). Under physiological conditions, Cis/Socs proteins may function in a GH-dependent negative feedback manner; their importance in regulating postnatal growth has been confirmed in transgenic and null mice. Transgenic overexpression of *Cis* resulted in impaired STAT5 phosphorylation and stunted growth, whereas the *Socs-2* null mouse exhibited significantly increased size compared with wild-type (WT) littermates (24, 25). Because of embryonic or perinatal lethality, *Socs-1* and *Socs-3* null mice have not been informative with respect to regulation of postnatal growth (21, 31). Several inflammatory cytokines including TNF-α and IL-6 can upregulate *Cis* and *Socs 1–3* hepatic RNA expression (8). This may occur via STAT5 (*Cis*) or STAT3 (*Socs-3*) cis elements in their respective promoters (1, 35). Recent reports (18, 28) have also identified posttranscriptional mechanisms of regulation involving control of protein translation, as well as mechanisms involving protein stability and proteasomal deg-

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radation. However, the relative importance of cytokine-dependent alterations in Ghr or Cis/Socs 1–3 abundance in modulating hepatic GH signaling was not known.

Intraperitoneal LPS administration induces hepatic production of several inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and is established as an experimental model of the acute phase response (14–17). It has recently been reported that LPS administration reduced GH-dependent hepatic STAT5b activation in a time-dependent manner (4, 22). Moreover, LPS, IL-6, and, to a lesser extent, TNF-α, have each been shown to induce murine hepatic *Socs-1, -2,* and *-3* RNA expression (6, 8, 22). Importantly, in these studies, intact TNF signaling was not required for IL-6 to elicit maximal initial induction of *Socs-1, -2,* and *-3* RNA and SOCS-3 protein (6). Moreover, TNF-α, IL-1β, or IL-6 treatment of primary rat hepatocytes has reproduced the in vivo induction of *Socs* 1–3 RNA, and TNF-α and IL-1β have been shown to inhibit GH upregulation of IGF-I in vitro (8, 12, 34, 37). We hypothesized that IL-6 would be required for LPS to inhibit hepatic STAT5b activation by GH and that this would involve upregulation of Cis/Socs proteins. In this study, we have confirmed that inhibition of STAT5b activation by LPS is temporally associated with upregulation of Cis and Socs-3 and that this is dependent on intact IL-6 signaling.

MATERIALS AND METHODS

Materials. Eight-week-old male C57BL/6J WT, TNFR1 null (Tnfrsfla, cat. no. 002818) and IL-6 null (B6.129s6-IL6tm1Kopf, cat. no. 002650) mice were obtained from Jackson Laboratory (Bar Harbor, ME). LPS, *Escherichia coli* 026:B6, and human GH were obtained from Sigma (St. Louis, MO). STAT5b (cat. no. sc-835), STAT3-P04 (cat. no. sc-8059), CIS (cat. no. sc-1529), SOCS 1–3 (cat. no. sc-9023, 7007, and 7009), ACTIN (cat. no. sc-1615), and SH-PTP1 (cat. no. sc-287) polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A STAT5-P04 (phosphotyrosine 699 of STAT5b, cat. no. s4308) antibody was obtained from Sigma. The GHR AL-47 polyclonal antibody that recognizes the cytoplasmic domain of the GHR has previously been described (38). The Renaissance chemiluminescence kit was obtained from New England Nuclear (Wilmington, DE). The digoxigenin (DIG) EMSA kit was obtained from Boehringer-Mannheim (Indianapolis, IN). The NE-PER kit for preparation of liver protein was obtained from Pierce. TRIzol for preparation of RNA was obtained from GIBCO-BRL. An ELISA kit for determination of tissue TNF-α and IL-6 abundance was obtained from R&D Systems (Minneapolis, MN). Human GH levels in sera were determined in samples obtained 30 min after intraperitoneal administration of human GH utilizing a previously described assay (19).

Oligonucleotides. EMSA oligonucleotides were synthesized by GIBCO-BRL (Rockville, MD). The following synthetic oligonucleotide was used for the consensus STAT5 EMSA: STAT5s: 5′-GGACCTCTTGGAATTAAGGGA-′3. A double-stranded oligonucleotide for EMSA was generated by annealing this synthetic oligonucleotide with the complimentary sequence.

GH and LPS treatments. Endotoxemia was induced by a single intraperitoneal LPS injection (1 μg/g body wt). GH treatment consisted of a single intraperitoneal injection (3 mcg/gm or 0.3 μg/g body wt) administered 30 min before harvesting of livers. Control mice were injected with an equal volume of sterile saline. Three hours after LPS treatment, livers were harvested for preparation of nuclear and cytoplasmic proteins and total RNA. The study protocol was approved by the Yale Animal Care and Use Committee.

RT-PCR analysis of Ghr, Cis, and Socs-3 gene expression. Total RNA was extracted by using TRIzol (GIBCO Life Technologies, Gaithersburg, MD). Concentration and purity were confirmed by spectrophotometry (model DU-640B; Beckmann Instruments, Palo Alto, CA). RNA was stored at −80°C. The following primers and probes were used for the TaqMan RT-PCR assay (synthesized by PE Biosystems): mGhr: forward, 5′-GGATCTTTGTCAGGTCTTCTTAACCT-′3; reverse, 5′-CAAGAGTAGCTGGTGTAGCCTCACT-′3; probe, 5′-TGGCAGTCACCAGCAGCAGCATTTT-′3; mCis: forward, 5′-GCCTTCCCAGATGTGGTCA-′3; reverse, 5′-CCGGGTGT-CAGCTGCAC-′3; probe, 5′-CCTTGTGCAGCACTATGTGGC-CTCC-′3; mSocs-3: forward, 5′-GCTCCAAAAGCGAGTAC-CAGC-′3; reverse, 5′-AGTAGAATCCGCTCTCCTGCAG-′3; probe, 5′-TGGTGAACGCCGTGCGCAA-′3; rGAPDH: proprietary (PE Biosystems, Foster City, CA). Total Ghr transcripts were amplified as previously reported (12). TaqMan real-time quantitative PCR assay was performed on an ABI Prism 7700 Sequence Detection System, according to the manufacturer's protocol (Applied Biosystems). Amplification of GAPDH was performed to standardize the quantification of target cDNA, allowing relative quantitation by using the ABI Prism 7700 (SDS software). Briefly, 2.0, 1.0, 0.5, and 0.25 μl of synthesized mouse liver cDNA were amplified in triplicate for both GAPDH and each of the target genes to create a standard curve. Likewise, 2.0 μl of cDNA was amplified in triplicate in all isolated mouse liver samples for each primer/probe combination and GAPDH. Each sample was supplemented with both respective forward and reverse primers, fluorescent probe, and made up to 50 μl by using TaqMan Master-Mix (Applied Biosystems). Each target probe was amplified in a separate 96-well plate. All samples were incubated at 50°C for 2 min and at 95°C for 10 min and then cycled at 95°C for 15 s and 60°C for 1 min for 40 cycles. After the standard curves were calculated, the input amounts of cDNA of the unknown samples were calculated for all target probes and GAPDH. After the input amount of the probe sample was normalized to the GAPDH expression, the relative amount of the expressed RNA species in each unknown sample was calculated.

Preparation of nuclear and cytoplasmic proteins, EMSA, and immunoblot analysis. Nuclear and cytoplasmic proteins were prepared from mouse liver utilizing the NE-PER kit as per manufacturer's recommendations (Pierce). Oligonucleotide probes were end-labeled, and EMSA was performed utilizing the DIG-EMSA kit as per manufacturer's recommendations (Boeringher-Mannheim). In supershift assays, 2 μg of polyclonal antibody were added to the gelshift reaction for 1 h after preincubation of the probe and nuclear proteins for 20 min on ice. Nuclear (20 μg) or cytosolic (40–50 μg) proteins were also loaded onto 7.5 or 12% SDS-PAGE (Bio-Rad, Hercules, CA) and subjected to electrophoresis and electrotransfer onto nitrocellulose membranes. For the Ghr immunoblot, cytoplasmic proteins (100 μg) were immunoprecipitated by using Sepharose A beads before immunoblotting. Briefly, protein samples were suspended in 100 μl immunoprecipitation buffer [20 mM HEPES, pH 7.5, 1% IDEAL CA-630 (Sigma), 10% glycerol, 2.5 mM EGTA, 2.5 mM EDTA, 1 μg/ml PMSF, leupeptin, aprotinin, and pepstatin, and 1 mM NaF]. Samples were then incubated with 3 μl Ghr antibody or preimmune rabbit serum with mixing at 4°C for 6 h, followed by incubation with 20-μl protein A beads

(Pierce) with end-over-end mixing at 4°C overnight. Protein bead complexes were collected by centrifugation at 1,000 *g* at 4°C for 5 min and then washed three times with 500 μl immunoprecipitation buffer. Proteins were eluted by boiling samples for 5 min in 40 μl 1× sample buffer (75 mM Tris, pH 6.8, 2.5% SDS, 12.5% glycerol, and 3% 2-mercaptoethanol, containing bromophenol blue dye). Uniformity of protein loading and transfer was assessed by using Ponceau staining and by reprobing immunblots for SH-PTP1 (nuclear proteins) or ACTIN (cytosolic proteins). Nitrocellulose membranes were blocked overnight at 4°C in 20 mM Tris·HCl, 150 mM NaCl, 5% nonfat dry milk, 0.1% Tween-20 [Tris saline (TS) complete]. Blots were incubated at room temperature for 1 h with STAT5b, STAT5-P04, STAT3-P04, GHR AL-47, ACTIN, or SH-PTP1 (1:1,000) or CIS or SOCS-1, 2, or 3 (1:200) antibodies in TS complete. Blots were washed and then incubated with anti-rabbit horseradish peroxidase conjugate antibody (1:2,000 to 1:5,000; Santa Cruz Biotechnologies) for 1 h at room temperature. Immune complexes were detected by using the New England Nuclear Chemiluminescence Plus Western blotting kit and Kodak Biomax light film. Band signal intensity was quantified by densitometry (Molecular Dynamics, Sunnyvale, CA).

ELISA. Liver tissue TNF-α or IL-6 abundance was determined by using cytoplasmic protein extracts and an ELISA kit as per manufacturer's recommendations (R&D Systems).

Statistical analysis. Data were expressed as the means ± SD of experiments with at least three independent treatments per group. Differences among experimental groups were analyzed by ANOVA. Where there were differences among the groups ($P < 0.05$), these were subjected to post hoc comparisons by using the unpaired Students *t*-test. $P < 0.05$ was considered to be significant.

RESULTS

LPS pretreatment inhibits STAT5b activation and DNA binding. Recent studies have demonstrated that LPS administration would inhibit GH activation of STAT5b in rat liver (4, 22, 36). This was time dependent with maximal inhibition of STAT5b activation observed from 3–4 h after the administration of a single intraperitoneal dose of LPS. Intrahepatic production of several cytokines, which may modulate GH signaling, including TNF-α, IL-1β, and IL-6, are increased within 1–3 h after LPS administration to WT mice. As a basis for studies in TNFR1 and IL-6 null mice, we initially determined whether LPS pretreatment would inhibit STAT5b activation in C57BL/6 mice. GH ± LPS was administered to WT mice, and liver nuclear proteins were isolated. Total and tyrosine phosphorylated STAT5b nuclear levels were determined by immunoblotting. The primary GH dose used in this study was in the range of what has typically been reported in the literature (e.g., from 1 to 3 μg/g) in other studies examining the effect of LPS on hepatic GH signaling. For example, Mao et al. (22) used 1.5 μg/g GH and 1 μg/g LPS and demonstrated upregulation of CIS and SOCS-3 RNA, which temporally preceded inhibition of STAT5 activation. The authors stated that the GH dose used represented a 10-fold higher amount than would be expected to yield a maximal hepatic response in intact rodents. They reasoned that a supramaximal GH dose was more appropriate for this type of study, to ensure that observed changes in signaling were not due to altered hormone delivery to tissue receptors. Two other recent papers examining LPS regulation of liver GH action used similar doses i.e., Defalque et al. (12) used 3 μg/g GH, whereas Wang et al. (36) used 1.5 μg/g GH.

As shown in Fig. 1*A*, either high (3 μg/g)- or low (0.3 mcg/gm)-dose GH treatment (for 30 min) significantly increased STAT5b nuclear levels in WT mice. LPS pretreatment (for 3 h) inhibited STAT5 activation in either case. Low-dose GH administration yielded a

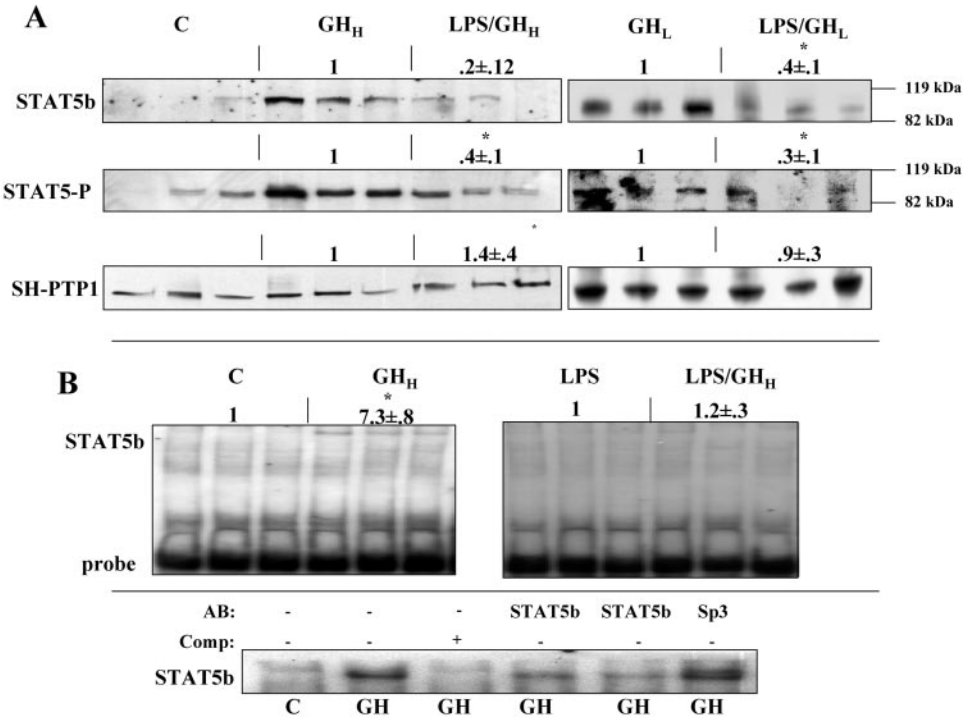

Fig. 1. LPS pretreatment inhibits STAT5b activation and DNA binding. Wild-type (WT) mice were treated with growth hormone {GH; 3 μg/g [high-dose GH (GH_H)] or 0.3 μg/g [low-dose GH (GH_L)} ip 30 min before death} ± LPS (1 μg/g ip 3 h before death) and liver nuclear proteins were isolated. *A*: immunoblots were performed by using polyclonal STAT5B, STAT-P04, or SH-PTP1 antibodies. *B*: EMSA was performed by using these proteins and a consensus STAT5 oligonucleotide ± a polyclonal STAT5B antibody. The mean signal intensity ± SD as determined by densitometry compared with treatment with GH alone (*A*) or control or LPS alone (*B*) for each group is shown; $^*P < 0.05$ by ANOVA, $n = 3$–6. C, control; STAT5-P, polyclonal antibody recognizing tyrosine phosphorylated STAT5; AB, antibody used for supershift; Comp, 100× excess unlabeled STAT5 oligonucleotide used for competition. Sp3, polyclonal antibody used as nonspecific control for supershift.

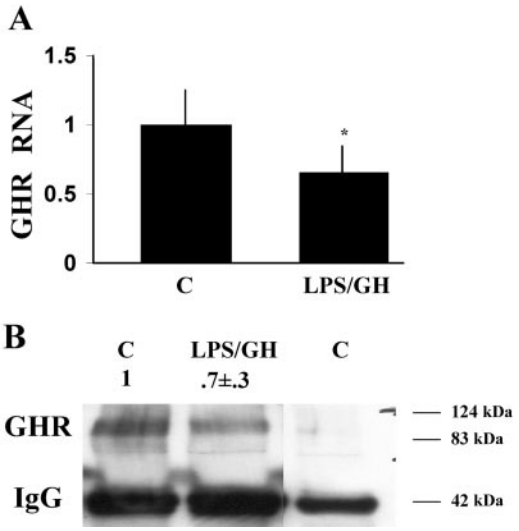

Fig. 2. Ghr abundance is modestly reduced by short-term LPS treatment. WT mice were treated with GH (3 μg/g ip 30 min before death) and LPS (1 μg/g ip 3 h before death) and liver protein and total RNA were isolated. *A*: real-time PCR was performed for total liver GHR transcripts. The mean GHR RNA level normalized to GAPDH and the associated range of values is shown. *B*: liver protein was immunoprecipitated by using the AL-47 polyclonal Ghr antibody or preimmune rabbit serum and immunoblots were performed by using the Ghr antibody. A representative blot is shown together with the mean signal intensity ± SD as determined by densitometry for each group; $^*P < 0.05$ by ANOVA, $n = 3$. The position of molecular weight markers is shown. The result with preimmune serum is shown in *lane 3*. Ghr, 110 kDa Ghr band; IgG, nonspecific band corresponding to the IgG heavy chain.

serum level of human GH of 92 ± 30 ng/ml in WT mice and 108 ± 37 ng/ml in IL-6 null mice. Therefore, it is unlikely that the inhibition (WT mice) or preservation (IL-6 null mice, see Fig. 5) of GH action observed in LPS-pretreated mice was related primarily to the dose of GH used, but rather to the dependence of the postreceptor inhibitors CIS and SOCS-3 on intact IL-6 signaling. Taken together, these data demonstrated that LPS pretreatment would inhibit GH activation of STAT5b in liver.

On activation by JAK2 in response to GH, phosphorylated STAT5b translocates to the nucleus and binds to cis elements in the promoters of target genes. We examined binding of nuclear proteins from WT mice to a STAT5 consensus oligonucleotide to determine whether the observed differences in STAT5b phosphorylation were associated with alterations in DNA binding. EMSA confirmed that GH-dependent STAT5b DNA binding was significantly inhibited by LPS pretreatment in WT mice (see Fig. 1*B*). The addition of a polyclonal STAT5b antibody reduced the intensity of the induced complex and confirmed that it contained the STAT5b protein (see Fig. 1*B*). These data demonstrated that LPS pretreatment did inhibit GH-dependent upregulation of STAT5b DNA binding in WT mice.

Ghr protein abundance is modestly reduced by short-term LPS treatment. One mechanism that could account for the LPS-dependent inhibition of GH signaling would involve downregulation of the Ghr itself. Two recent reports (12, 22) utilizing short-term (3–6 h) low (1 μg/g)- or high (7.5 μg/g)-dose LPS administration have demonstrated either no change in hepatic GHR protein levels or a significant reduction in GH binding, respectively. We have recently reported (13) that longer-term (12 h) treatment with low-dose LPS (1 μg/g) reduced hepatic *Ghr* gene expression via TNF-α-dependent inhibition of binding of Sp1/Sp3 transcription factors to adjacent cis elements in the *Ghr* gene L2 promoter. We therefore examined *Ghr* gene expression in this model. As shown in Fig. 2*A*, short-term LPS treatment led to a modest reduction in *Ghr* RNA levels in WT mice. Immunoblots were then performed to determine whether the observed reduction in RNA expression was associated with an alteration in Ghr abundance. As shown in Fig. 2*B*, total Ghr protein abundance was also modestly reduced, by ~30%, although this difference was not statistically significant ($n = 3$). Precipitation with preimmune rabbit serum confirmed that the identified Ghr complex was specific (see *lane 3* of Fig. 2*B*). Therefore, the observed reduction in STAT5 activation was likely due primarily to induction of postreceptor signaling inhibitors.

Induction of Cis and Socs-3 protein is temporally associated with inhibition of STAT5b activation. Several reports have now detailed postreceptor mechanisms by which Cis and Socs 1–3 can inhibit GH activation of STAT5b in liver (27). We therefore determined Cis and Socs 1–3 protein expression in response to LPS pretreatment in WT mice to determine whether this was associated with inhibition of STAT5b activation. As shown in Fig. 3, Cis and Socs-3 proteins were significantly upregulated by LPS pretreatment. Interestingly, despite prior reports demonstrating LPS-dependent upregulation of *Socs*-1 and *Socs*-2 RNA expression, an increase in Socs-1 or Socs-2 protein was not observed (data not shown) (6, 8). These data indicated that upregulation of Cis and Socs-3 was likely involved in the observed inhibition of STAT5b activation by LPS.

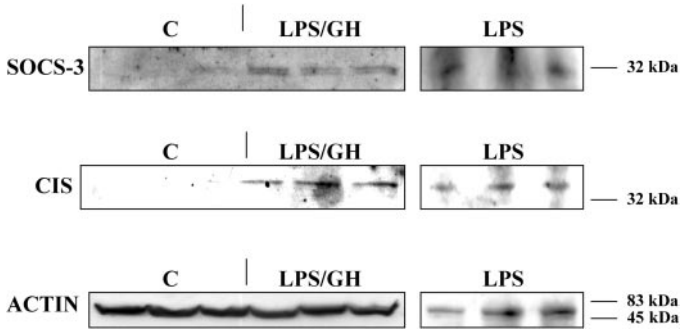

Fig. 3. LPS upregulation of Cis and Socs-3 protein is temporally associated with inhibition of STAT5b activation. WT mice were pretreated with GH (3 μg/g ip 30 min before death) ± LPS (1 μg/g ip 3 h before death) and liver protein was isolated. Immunoblots were performed by using these proteins and Cis, Socs-3, or ACTIN polyclonal antibodies. The position of molecular weight markers is shown.

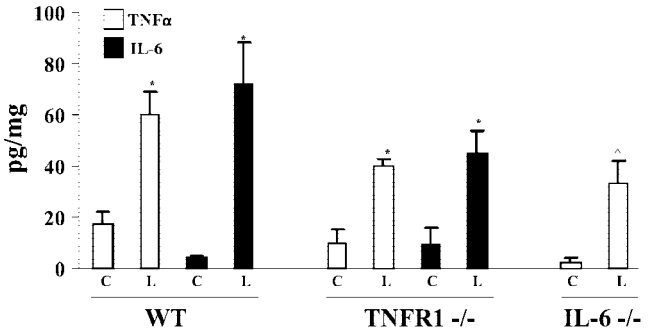

Fig. 4. LPS upregulation of TNF-α and IL-6 in liver. ELISA was used to determine TNF-α or IL-6 abundance in liver protein isolated 3 h after LPS administration (1 μg/g ip) to WT, TNF receptor 1 (TNFR1) null, or IL-6 null mice. *$P < 0.05$ by ANOVA vs. control of the same genotype; ^$P < 0.05$ by ANOVA vs. control of the same genotype and vs. WT after LPS administration.

LPS upregulation of hepatic TNF-α and IL-6 abundance. Several prior studies have reported upregulation of TNF-α and IL-6 in serum after intraperitoneal LPS administration to WT and TNFR1 or IL-6 null mice (17, 29). Because both TNF-α and IL-6 have previously been shown to upregulate Cis and Socs-3 in liver, we determined abundance of these cytokines in mice after LPS administration. Serum levels of these cytokines are typically assumed to reflect local hepatic expression in this acute-phase model. For TNFR1 null mice, LPS administration was shown to yield a two- to threefold reduction in upregulation of serum IL-6 compared with WT mice (29). Conversely, for IL-6 null mice, LPS administration was shown to yield a threefold higher serum level of TNF-α compared with WT mice (17). These alterations in LPS-dependent cytokine expression compared with WT could contribute to differences in acute-phase gene regulation. However, it is possible that serum levels of TNF-α and IL-6 could also reflect synthesis in other tissues stimulated by intraperitoneal LPS administration, such as peritoneal macrophages. Therefore, we utilized ELISA to determine hepatic tissue abundance of TNF-α and IL-6 in WT and TNFR1 and IL-6 null mice after LPS administration. As shown in Fig. 4, LPS administration upregulated liver TNF-α (60 ± 9.2 pg/mg) and IL-6 (71.5 ± 16 pg/mg) abundance in WT mice. Both of these cytokines were also significantly upregulated by LPS in TNFR1 null mice (TNF-α, 40 ± 2.8 pg/mg; IL-6, 44.6 ± 9.3 pg/mg). Although these were lower than in WT mice, this difference was not statistically different. LPS administration increased TNF-α abundance to 33 ± 9.9 pg/mg in IL-6 null mice. This difference was significantly less than in WT mice. These data confirmed that LPS administration upregulated hepatic TNF-α and IL-6 abundance in WT mice. Moreover, IL-6 upregulation in TNFR1 null mice and TNF-α upregulation in IL-6 null mice were reduced by ~50% compared with WT mice. Studies were then performed to determine the relative importance of TNF-α vs. IL-6 in upregulating Cis/Socs-3 and inhibiting STAT5 activation.

IL-6 mediates LPS-dependent inhibition of STAT5b activation. A recent report has described a temporal association between LPS-dependent upregulation of IL-6 and *Socs-3* and inhibition of GH activation of IGF-I (36). However, whether inflammatory cytokines would have redundant effects on GH signaling in vivo, or whether a single cytokine, such as TNF-α or IL-6 would be required for LPS inhibition of STAT5b activation was not known. To test this, the effect of LPS pretreatment on hepatic STAT5b activation in TNFR1 and IL-6 null mice was determined. As shown in Fig. 5, GH treatment (for 30 min) significantly increased both total and tyrosine phosphorylated STAT5b nuclear levels in TNFR1 or IL-6 null mice. LPS pretreatment (for 3 h) inhibited STAT5b activation in TNFR1 null mice to a degree comparable with that seen in WT mice (Fig.

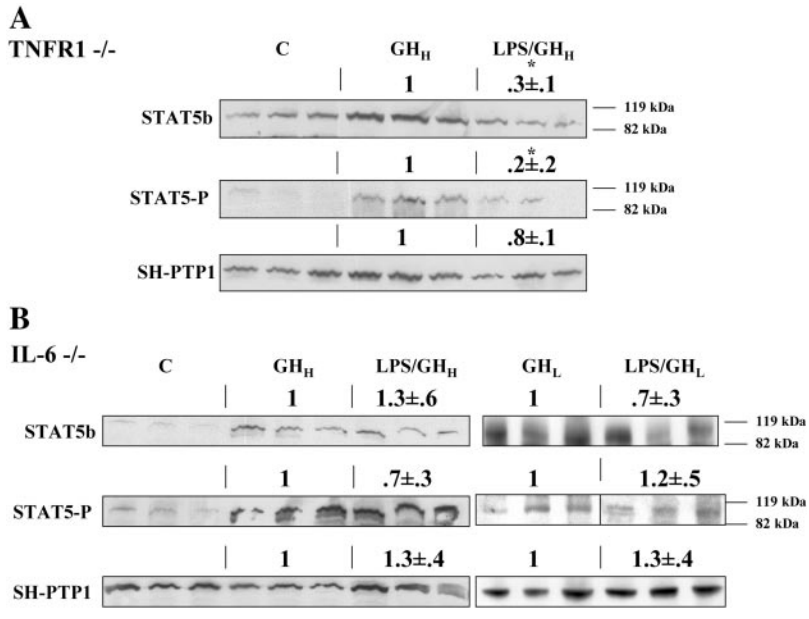

Fig. 5. IL-6 mediates LPS-dependent inhibition of STAT5b activation. TNFR1 and IL-6 null mice were treated with GH [3 μg/g (GH_H) or 0.3 μg/g (GH_L) ip 30 min before death] ± LPS (1 μg/g ip 3 h before death) and liver nuclear proteins were isolated. Immunoblots were performed with these proteins and polyclonal STAT5B, STAT-P04, or SH-PTP1 antibodies. The mean signal intensity ± SD as determined by densitometry compared with treatment with GH alone for each group is shown; *$P < 0.05$ by ANOVA, $n = 3$–6. The position of molecular weight markers is shown. STAT5-P, polyclonal antibody recognizing tyrosine phosphorylated STAT5B.

1*A*). In contrast to this, STAT5 activation was preserved despite LPS pretreatment in IL-6 null mice. This was not dependent on the dose of GH used, because STAT5 activation was preserved with either high (3 μg/g)- or low (0.3 μg/g)-dose GH. These data demonstrated that LPS inhibition of STAT5b activation required intact IL-6 signaling. Because Cis and Socs-3 have been shown to inhibit GH-dependent activation of STAT5, we then determined whether upregulation of these proteins differed between LPS-treated TNFR1 and IL-6 null mice.

IL-6 signaling is required for upregulation of Cis and Socs-3. Several inflammatory cytokines including TNF-α and IL-6 have been shown to upregulate *Cis* and *Socs* 1–3 RNA expression (8). However, a recent in vivo study (6) of hepatic regeneration demonstrated that IL-6 alone could induce *Cis* or *Socs 1–3* RNA expression in the absence of intact TNF signaling, whereas the converse was not true. However, the duration of *Socs-3* induction was significantly less after the administration of IL-6 alone vs. partial hepatectomy, in which multiple cytokines including TNF-α were also induced (6). Whether IL-6-dependent upregulation of Cis and Socs-3 was necessary and sufficient to acutely inhibit hepatic GH signaling was not known. As shown in Figs. 3 and 6, Cis and Socs-3 proteins were upregulated by LPS in WT and TNFR1 null mice (note that for Socs-3 upregulation by LPS/GH in TNFR1 null mice, $n = 2$). By comparison, Cis or Socs-3 upregulation in LPS-treated IL-6 null mice was either not detected or significantly reduced compared with TNFR1 null mice. Taken together, these data indicated that IL-6 inhibited hepatic GH signaling via upregulation of Cis and Socs-3 protein abundance. We then determined whether the observed alterations in Cis and Socs-3 protein abundance were associated with differences in RNA expression.

IL-6 mediates LPS activation of STAT3 and upregulation of Socs-3 RNA expression. In most experimental systems reported to date, cytokine upregulation of *Cis* or *Socs-3* in hepatocytes has been reported at the RNA level, and is assumed to correlate with corresponding changes in protein abundance (8). Recently, the administration of IL-6 alone has been shown to upregulate both Socs-3 RNA and protein in liver (6). Interestingly, whereas TNF-α administration was also shown to upregulate *Socs-3* RNA expression (although to a fourfold lesser degree) in that study, no Socs-3 protein was detected. Therefore, we determined *Cis* and *Socs-3* RNA expression in TNFR1 and IL-6 null mice after LPS administration. As shown in Fig. 7*A*, *Socs-3* RNA expression was upregulated to a significantly greater degree in LPS-treated TNFR1 null (16 ± 2 ng) vs. IL-6 null (4.1 ± 2.5 ng) mice, although it was upregulated in both vs. controls. By comparison, absolute *Cis* RNA expression did not differ between LPS-treated TNFR1 null (8.4 ± 4.6 ng) and IL-6 null (9.8 ± 4.2 ng) mice, although the upregulation compared with control was greater for the TNFR1 null mice (ninefold vs. twofold). These data indicated that the observed difference in Socs-3 protein abundance between LPS-treated TNFR1 null and IL-6 null mice was due to a difference in RNA expression, whereas the observed difference in Cis protein abundance might also be due to effects of IL-6 on Cis protein translation.

Positive-acting STAT cis elements have been identified in the mouse *Cis* and *Socs-3* promoters (1, 23, 35). For *Cis,* either STAT3 or STAT5A/B have been shown to bind adjacent cis elements under different experimental conditions, whereas STAT1 and STAT3 has been shown to bind a proximal *Socs-3* promoter element. These STAT elements have, in turn, been shown to mediate cytokine upregulation of promoter activity. We therefore examined STAT3 phosphorylation and

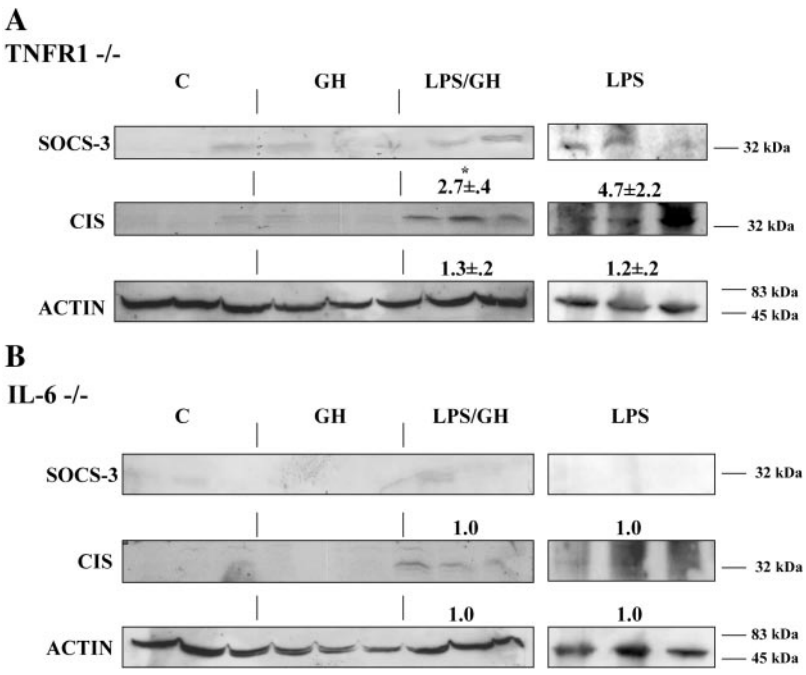

Fig. 6. IL-6 signaling mediates upregulation of Cis and Socs-3 protein. TNFR1 and IL-6 null mice were treated with GH (3 μg/g ip 30 min before death) ± LPS (1 μg/g ip 3 h before death) and liver cytoplasmic protein was isolated. Immunoblots were performed by using these proteins and Cis, Socs-3, or ACTIN polyclonal antibodies. The mean Cis or Socs-3 protein abundance ± SD for LPS-treated TNFR1 null compared with IL-6 null mice as determined by densitometry is shown. $^*P < 0.05$ by ANOVA for TNFR1 null vs. IL-6 null mice, $n = 3$.

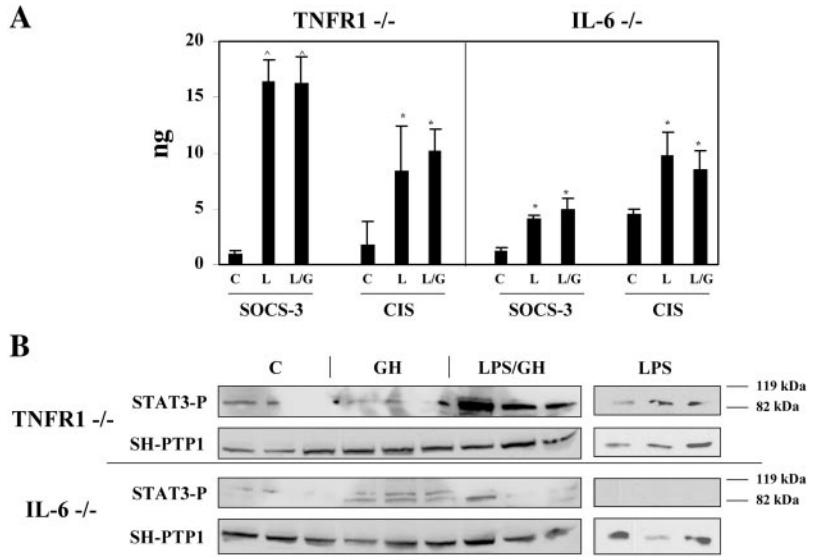

Fig. 7. IL-6 upregulation of *Cis* and *Socs-3* RNA expression is associated with STAT3 activation. TNFR1 and IL-6 null mice were treated with GH (3 μg/g ip 30 min before death) ± LPS (1 μg/g ip 3 h before death) and liver total RNA and nuclear proteins were isolated. *A*: *Cis* and *Socs-3* RNA expression were determined by using real time PCR. *B*: immunoblots were performed by using liver nuclear proteins and STAT3-P04 and SH-PTP1 polyclonal antibodies. $^*P < 0.05$ by ANOVA vs. control of same genotype; $^\wedge P < 0.05$ vs. control of same genotype and vs. LPS-treated IL-6 null mice. STAT3-P04, polyclonal antibody recognizing tyrosine phosphorylated STAT3.

nuclear abundance under conditions in which *Cis* and *Socs-3* RNA expression was upregulated. As shown in Fig. 7*B*, LPS induced tyrosine phosphorylation and nuclear translocation of STAT3 in TNFR1 but not IL-6 null mice. This was temporally associated with upregulation of *Cis* and *Socs-3* RNA expression and inhibition of STAT5 in TNFR1 null mice.

DISCUSSION

Signal-transduction pathways that mediate GH-dependent activation of STAT5 transcription factors in liver have been extensively studied and are now well defined (9). Until recently, the factors that negatively regulated GH signaling were less well understood (21, 27). In particular, whereas evidence of GH resistance had been observed in inflammatory diseases and in experimental models of inflammation, the specific factors that mediate cellular resistance to GH had not been identified. Identification of these factors will be a critical step in designing therapies to selectively restore GH signaling in inflammatory conditions. In this study, we have demonstrated that IL-6 upregulation of Cis and Socs-3 in liver can significantly inhibit GH activation of STAT5b.

The relative importance of TNF-α vs. IL-6 in suppressing GH signaling in inflammatory conditions has been difficult to determine. Transgenic mice that overexpress either TNF-α or IL-6 exhibit stunted growth with evidence of GH resistance and reduced IGF-I synthesis (11, 20). The overproduction of TNF-α and IL-6 that occurs in experimental colitis has also been associated with GH resistance; this was partially reversed by treatment with either anti-TNF-α or anti-IL-6 antibodies, although anti-IL-6 was more effective (2, 3). The role that alterations in Ghr or Cis/Socs protein abundance may play in causing inflammatory GH resistance has not been determined, although potential mediators, such as Socs-3, have been shown to be upregulated in patients with inflammatory bowel disease and in experimental colitis (32).

GH signaling could be inhibited by cytokines at the receptor or postreceptor level. We have recently reported (13) that LPS administration led to a 70% reduction in hepatic *Ghr* gene expression 12 h later and that this was due to TNF-dependent reduction in DNA binding of *Ghr* gene promoter Sp1/Sp3 transactivators. In a model utilizing an eightfold higher dose of LPS than in the present study, reduced IGF-I activation associated with a reduction in total liver GH binding was demonstrated 6 h after treatment (12). However, another group, (22) which used the same LPS dose as in the present study for 4 h, did not observe a reduction in Ghr abundance. Importantly, in a recent report (30) describing GH resistance in experimental chronic uremia, although hepatic *Ghr* RNA levels were reduced, this was not associated with a reduction in Ghr protein abundance or GH binding. In the present study, in which samples were isolated 3 h after low-dose LPS administration, we observed a modest (~30%) reduction in *Ghr* RNA expression and protein expression. Therefore, it was likely that primarily postreceptor mechanisms accounted for the observed differences in STAT5b activation in this model. However, downregulation of the Ghr may play a significant role with longer-term exposure to LPS in chronic inflammatory conditions. This is currently being investigated.

A significant advance in understanding postreceptor modulation of GH signaling has come with cloning of members of the SOCS family including Cis and Socs 1–3 (21). In vitro studies (27, 28) have demonstrated that each of these proteins can inhibit GH signaling by preventing STAT5b phosphorylation in association with the activated GHR:JAK2 complex. Stunted growth in *Cis* transgenic mice and the high-growth phenotype of the *Socs-2* null mouse have confirmed their importance in regulating postnatal growth (24, 25). Recent studies (6) have demonstrated that *Cis/Socs 1–3* are positive acute-phase hepatic genes that are potently upregulated by LPS and inflammatory cytokines, in particular IL-6. Moreover, a temporal

association was observed between maximal inhibition of GH activation of STAT5b or IGF-I by LPS and upregulation of IL-6 protein and *Socs-3* RNA expression (22, 36). Therefore, the *Cis*/*Socs* genes represented good candidates for inflammatory inhibitors of GH action. However, direct evidence for this in vivo was lacking, and regulation of hepatic Cis and Socs 1–3 protein abundance by LPS had not been characterized.

In the present study, GH-dependent STAT5b activation was abolished in WT or TNFR1 null mice in which LPS activated STAT3 and upregulated CIS and SOCS-3 RNA and protein. STAT5b activation was preserved in IL-6 null mice in which STAT3 activation was prevented and CIS/SOCS-3 protein were not upregulated. Taken together, these data support a molecular mechanism of GH resistance in which IL-6 activation of STAT3 leads to upregulation of *Cis* and *Socs-3*, and inhibition of STAT5b phosphorylation by the GHR:JAK2 complex. This is consistent with a recent report (36) using a similar model of the LPS-induced hepatic acute-phase response, in which inhibition of IGF-I upregulation by GH was temporally associated with elevated IL-6 levels and *Socs-3* RNA expression. Regulation of the murine *Cis* and *Socs-3* genes by cytokines and STAT transcription factors has been characterized in several prior reports (1, 23, 35). STAT5a has been identified as a critical regulator of *Cis*; however, in the present study, upregulation of CIS occurred under conditions in which STAT3, but not STAT5, was activated (35). This is consistent with a recent report (10) in which GH-dependent upregulation of *Cis* expression was preserved in STAT5b null mice, indicating that alternate transcription factors, potentially including STAT3, can also upregulate *Cis*. The STAT1/STAT3 cis element that has been characterized in the mouse *Socs-3* proximal promoter could mediate the SOCS-3 upregulation we observed (1). However, although IL-6 has been shown to upregulate the rat *Socs-3* promoter in hepatocytes, this effect was not mapped to the known STAT1/STAT3 elements (26). Therefore, regulation of *Cis* or *Socs-3* expression by GH or IL-6 and associated STAT transcription factors is complex and appears to be dependent on the specific tissue and experimental conditions used. It should be noted that studies utilizing hepatocytes in culture have also identified a potential role for IL-1β in upregulating *Socs-3* RNA and inhibiting GH-induced expression of the *ALS* gene (5). Taken together, these data add to the growing body of evidence supporting a critical role for CIS/SOCS proteins in modulating GH signaling in inflammatory conditions (22). This may provide for several therapeutic options, including targeted blockade of specific cytokines or individual CIS/SOCS proteins, in addressing complications of inflammatory GH resistance.

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