Interleukin-6 inhibits growth hormone-mediated gene expression in hepatocytes

Tamer A. Ahmed,1 Mark D. Buzzelli,1 Charles H. Lang,2 John B. Capen,1 Margaret L. Shumate,1 Maithili Navaratnarajah,1 Murali Nagarajan,1 and Robert N. Cooney1,2

Departments of 1Surgery and 2Cellular and Molecular Physiology, The Pennsylvania State University-College of Medicine, Hershey, Pennsylvania

Submitted 28 November 2006; accepted in final form 23 March 2007

Ahmed TA, Buzzelli MD, Lang CH, Capen JB, Shumate ML, Navaratnarajah M, Nagarajan M, Cooney RN. Interleukin-6 inhibits growth hormone-mediated gene expression in hepatocytes. Am J Physiol Gastrointest Liver Physiol 293: G1793–G1803, 2007. First published March 29, 2007; doi:10.1152/ajpgi.00547.2006.—During systemic inflammation, the liver becomes unresponsive to growth hormone (GH), resulting in decreased plasma insulin-like growth factor-I (IGF-I) with concomitant reductions in lean body mass. Transgenic mice that overexpress IL-6 also demonstrate impaired growth and decreased IGF-I. To determine whether IL-6 directly inhibits GH-inducible gene expression, CWSV-1 hepatocytes were incubated with IL-6 (10 ng/ml), then stimulated with recombinant human GH (500 ng/ml, 18 h). The increase in IGF-I and serum protease inhibitor 2.1 (Spi 2.1) mRNA in GH-treated cells was inhibited by treatment with IL-6 for 24 h. To investigate potential mechanisms, we examined the effects of IL-6 on GH receptor (GHR) expression and GH signaling via the JAK/signal transducer and activator of transcription (STAT) and MAP kinase pathways. Incubation of cells with IL-6 (10 ng/ml, 24 h) had no effect on GHR abundance or signaling proteins JAK2, STAT5b, and ERK1/2. Although GH transiently increased (2- to 5-fold) the tyrosine phosphorylation of GHR, JAK2, STAT5b, and ERK1/2, IL-6 did not alter these phosphorylation events. However, nuclear protein from IL-6-treated cells demonstrated reduced STAT5 DNA binding (by EMSA) at 15 min (−20%) and 60 min (−43%) after GH stimulation. To determine whether IL-6 inhibits GH-inducible promoter activity, CWSV-1 cells were transfected with Sp1 2.1 or prolactin receptor promoter luciferase vectors, incubated with or without IL-6, then stimulated with GH. The induction of both Sp1 2.1 (7.5-fold) and prolactin receptor (4-fold) promoter activity by GH was inhibited by IL-6. In summary, IL-6 mediates hepatic GH resistance by a time-dependent inhibition of GH-inducible promoter activity that is associated with reductions in STAT5 DNA binding.

IN HEALTHY INDIVIDUALS, PULSATILE secretion of growth hormone (GH) by the pituitary stimulates the synthesis and secretion of insulin-like growth factor-I (IGF-I) by liver and other tissues. Although somatic growth was originally thought to be controlled by hepatic IGF-I synthesis alone, direct effects of GH and the paracrine secretion of IGF-I by other tissues appears to be important as well (35). Normally, the liver is the major site of synthesis for circulating IGF-I, primarily in response to GH stimulation and nutrient intake (35, 55). However, during systemic inflammation hepatic IGF-I mRNA and plasma levels of IGF-I are coordinately decreased despite normal or elevated GH levels (1–3, 7, 17–21, 52). The inflammatory cytokines tumor necrosis factor (TNF), interleukin (IL)-1, and more recently IL-6 have been implicated in the pathogenesis of hepatic GH resistance (2, 17–21, 26, 33, 34).

Whereas TNF and IL-1 inhibit GH signaling and/or gene expression in cultured hepatocytes, the evidence implicating IL-6 as a potential mediator of hepatic GH resistance is derived primarily from in vivo studies (2, 15, 17, 37, 50). In a rat model of colitis-induced growth failure, a sixfold increase in plasma IL-6 was observed in conjunction with a 65% reduction in circulating IGF-I and a threefold increase in GH (2). Decreased nutrient intake and systemic inflammation appear to contribute equally to the impaired growth in the colitis group (2). Administration of anti-IL-6 antibodies to the colitis group improved growth and plasma IGF-I levels without affecting food intake or intestinal inflammation, suggesting IL-6-induced hepatic GH resistance as a cause of growth failure (50). Overexpression of IL-6 in transgenic mice is also associated with impaired growth and reductions in circulating IGF-I levels (15). Neutralization of IL-6 overexpression in transgenic mice with a monoclonal antibody against the IL-6 receptor significantly improved growth in these animals (15). Food intake and plasma GH levels were normal in the IL-6 transgenic mice, whereas hepatic IGF-I mRNA levels were decreased (37). Collectively, these results suggest that overexpression of IL-6 causes the liver to become unresponsive or resistant to GH, resulting in decreased IGF-I synthesis and secretion.

The current study is the first to examine potential mechanisms for IL-6-mediated GH resistance in a cell culture model using CWSV-1 hepatocytes. Hepatic GH resistance may be caused by decreased expression of the GH receptor (GHR), impaired GH signaling, or reductions in GH-inducible gene expression. Normally, the binding of GH to the transmembrane GHR causes receptor dimerization and initiates GH signaling (30, 31). The GHR-GHR2 complex is constitutively associated with JAK2, a tyrosine kinase that phosphorylates itself and the janus kinase/signal transducer and activator of transcription (STAT) and mitogen-activated protein kinase signaling pathways in liver (8, 56, 65). The JAK2-STAT5 pathway is activated protein kinase pathway; insulin-like growth factor-I; serine protease inhibitor 2.1; tumor necrosis factor (TNF), interleukin (IL)-1; and more recently IL-6 have been implicated in the pathogenesis of hepatic GH resistance (2, 17–21, 26, 33, 34).
particularly important in regulating IGF-I synthesis (14, 43, 46). Studies in STAT5b knockout mice suggest that STAT5 is required for basal and GH-inducible expression of hepatic IGF-I (14). Animals with impaired STAT5 expression demonstrate a 50% reduction in circulating IGF-I and impaired growth, presumably as a result of reductions in plasma IGF-I (14). STAT5 is recruited to the activated GHR-JAK2 complex, where it undergoes tyrosine phosphorylation by JAK2 (30, 31, 46). Phosphorylated STAT5 dimerizes and then translocates to the nucleus, where it binds to specific DNA sequences in the promoter region of GH-inducible “target genes,” referred to as γ-activated or GAS sequences, to activate gene transcription (24, 25, 30, 31, 46). Serine phosphorylation of STAT5 by the MAP kinase pathway influences the magnitude of STAT5 transcriptional activation as well (45).

The current study investigates the time course and potential mechanisms for the inhibitory effects of IL-6 on GH-inducible gene expression in CWSV-1 hepatocytes. The inhibitory effects of IL-6 on GH-inducible IGF-I and serine protease inhibitor (Spi) 2.1 expression were “time dependent,” requiring a 12- to 24-h incubation with IL-6 to manifest. IL-6 did not alter the abundance of GHR/signaling proteins or the time course of GH signaling via the MAP kinase or STAT5 pathways. In contrast, IL-6 attenuated GH-inducible STAT5 DNA binding and the activity of Spi 2.1 and prolactin receptor (PRLr) promoter constructs. Collectively, these results suggest that the inhibitory effects of IL-6 are time dependent and involve the regulation of GH-inducible promoter activity.

METHODS

Materials and plasmids. Recombinant human GH (rhGH; Pharmacia and Upjohn, Stockholm, Sweden) was used in all experiments. Rat IL-6 was obtained from R&D Systems (Minneapolis, MN). The plasmid containing the rat IGF-I cDNA was a kind gift from Peter S. Rotwein (Dept. of Biochemistry and Molecular Biology, Oregon Health & Science University School of Medicine, Portland, OR). The oligonucleotide sequence used as a probe to detect Spi 2.1 is described by Bergad et al. (5). Polyclonal GHR antibody was obtained from W. R. Baumbaugh (American Cyanamid, Princeton, NJ) and was used at a dilution of 1:250 (49). Rabbit polyclonal STAT5b antibody (sc-835, Santa Cruz Biotechnology, CA) and PY20 phosphotyrosine antibody conjugated with horseradish peroxidase (BD Transduction Laboratories, San Diego, CA) were used for immunoblot analyses. Polyclonal JAK2 antibody and rabbit anti-phospho-JAK2 were obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal p44/42 MAP kinase antibody and phospho-p44/42 MAP kinase antibody that recognize ERK1 and ERK2 were obtained from Cell Signaling, New England Biolabs (Beverly, MA).

The construction of the Spi 2.1 promoter luciferase construct (−1059 to +8) was previously described (1). The PRLr luciferase reporter construct was generated as follows. The 5′-flanking region of the PRLr exon (−999/+81), when expressed in relation to the transcription start site of the exon, was inserted into pGL3-Basic (Promega, Madison, WI) (23). Briefly, the PRLr region −999/+81 was amplified by PCR using custom-designed primers, then cloned into the pCR II-Blunt-TOPO vector (Invitrogen). After sequence

Fig. 1. Interleukin (IL)-6 inhibits the induction of insulin-like growth factor (IGF)-I mRNA by growth hormone (GH). Cells were treated with 10 ng/ml IL-6 for 4, 12, or 24 h, then stimulated with 500 ng/ml recombinant human GH (rhGH) for 18 h. Northern blot analysis was performed as described in METHODS. IGF-I mRNA corresponds to the 7.5-kb exon 1-derived transcript. Densitometry data for IGF-I mRNA were normalized to 18S rRNA message and were expressed as means ± SE. A: cells were treated with IL-6 for 4 h, followed by GH for 18 h. aP < 0.001 vs. control. B: cells were treated with IL-6 for 12 h, then GH for 18 h. aP < 0.001 vs. control; bP < 0.01 vs. GH alone. C: cells were treated with IL-6 for 24 h, followed by GH treatment. aP < 0.001 vs. control; bP < 0.01 vs. GH alone. Blots are representative of experiments performed 4–8 times.
verification by the Molecular Genetics Core Facility of the Section of Research Resources, Pennsylvania State College of Medicine, the PRLr promoter region was subcloned into the pGL3-Basic vector (PRLr-pGL3).

Cell culture experiments. CWSV-1 hepatocytes, obtained from Dr. Harriet C. Isom (Dept. of Microbiology and Immunology, College of Medicine, Pennsylvania State University), were cultured as previously described (1, 32, 52, 61, 64). CWSV-1 cells were grown in chemically defined RPMI (RPCD) medium for 48 h. IL-6-treated cells were incubated with 10 ng/ml IL-6 for 4 to 24 h, and then 500 ng/ml rhGH was added for the indicated time periods.

Northern blot analysis. The relative abundances of IGF-I and Spi 2.1 mRNA were determined by Northern blot analysis as previously described (1, 52, 63, 64). For IGF-I, an 800-bp XhoI-EcoRI fragment corresponding to the rat IGF-I cDNA containing exons 1, 3, 4, 5, and 6 was used as a probe (1, 52, 63, 64). In liver, the exon 1-derived 7.5-kb transcript of IGF-I mRNA represents the predominant (80%) IGF-I mRNA species (55). The oligonucleotide (5’/H11032-ACG ATG CTG AGC ACC C-3’/H11032) was used to measure Spi 2.1 expression (5). After exposure of the completed blots to film, the autoradiographs were scanned by using an HP ScanJet 5300C model scanner. Northern blots were stripped and reprobed with the 18S ribosomal subunit message to confirm uniform loading of RNA (52, 63, 64). Scans were analyzed by using Scion Image for Windows (National Institutes of Health). Data are reported as relative densitometry units after normalization to 18S rRNA message.

Preparation of cell lysates and isolation of nuclear protein. Cell lysates were prepared from cells grown in culture dishes, then placed on ice and rinsed three times with cold PBS. Lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1.0 mM EGTA, 10% glycerol, 1% Triton X-100, 100 mM NaF, 0.2 mM Na₃VO₄, 1 mM PMSF, and 10 μg/ml aprotinin) was added to the dishes that were then incubated at 4°C for 30 min. Lysates were cleared of nuclei by centrifugation at 10,000 rpm for 5 min. Supernatants were snap frozen in liquid nitrogen and were stored at −70°C (52, 64). Nuclear extracts were prepared by using the Active Motif nuclear extraction kit (Carlsbad, CA).

Western blot analysis and immunoprecipitation. For the detection of total protein, equal amounts of protein were electrophoresed on a 7.5% polyacrylamide gel and were transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA) by using standard electroblotting procedures. For the detection of phosphorylated proteins, cell lysates (100–500 μg) were immunoprecipitated and immunocomplexes were resolved by using SDS-PAGE (52, 64). Total and phosphorylated GHR and total and phosphorylated STAT5b were measured by Western blot as previously described (52, 64). For the detection of total and phosphorylated
JAK2, the protocol from Upstate Cell Signaling Solutions was followed. Total and phosphorylated ERK1/ERK2 were measured according to the manufacturer’s guidelines (Cell Signaling). Antibody reactions were visualized with the use of ECL-Plus (Amersham Pharmacia Biotech). The intensity of antibody reactions was analyzed by using Scion Image for Windows.

**EMSA.** EMSA was performed as previously described (52, 63, 64). Oligonucleotides complimentary to the rat β-casein promoter 5’-GGA CTT CTT GGA ATT AAG GGA-3’ were custom ordered and labeled independently with T4 kinase (Promega). Nuclear protein from CWSV-1 cells (5 µg) was used in a binding reaction containing 2 µg of poly(dI-dC), 0.5 ng of probe (50,000 cpm), and 1X binding buffer (10 mM Tris, pH 7.5, 4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl) (48). Reactions were incubated for 30 min at 25°C and then electrophoresed on a 5% Tris-borate-EDTA precast gel (Bio-Rad Laboratories, Hercules, CA) for 1–2 h at 100 V at room temperature. To supershift complexes, STAT5b antibody (sc-835 X; Santa Cruz Biotechnology) was added to the reactions and was incubated for 30 min at 25°C before the addition of the probe. Gels were dried and exposed to film.

**Transient transfection.** CWSV-1 cells were plated in RPCD medium at a density of 2 × 10⁶ cells/100-mm dish for 24 h before transfection. Cells were transfected with 0.5 µg Spi 2.1-pGL3 promoter construct with the use of the Effectene reagent (Qiagen) according to the manufacturer’s directions. In separate experiments, cells were transfected with 0.5 µg PRLr promoter luciferase reporter construct (PRLr-pGL3). Medium was removed 20 h after transfection, and the cells were plated with fresh medium in the presence or absence of IL-6 (10 ng/ml) for 4 or 24 h. Cells were then treated with GH (500 ng/ml) for 4 h. Cells were washed with PBS and were lysed in 900 µl 1X lysis buffer (Promega). Cell extracts (20 µl) were assayed for luciferase activity, and 2 µl of extract was used to determine total protein concentration. Luciferase activity was reported as relative light units, normalized to total protein concentration (luciferase units/µg protein), and as fold induction above baseline control. Each data point represents the mean of triplicate measurements for an experiment repeated at least three times.

**mRNA stability.** The transcriptional inhibitor 5,6-dichloro-β-D-ribofuranosyl benzimidazole (DRB, 72 µM; Calbiochem, La Jolla, CA) was used to examine the effects of IL-6 on the stability and natural decay kinetics of GH-induced IGF-I, Spi 2.1, and PRLr mRNA. CWSV-1 cells in the presence or absence of IL-6 (10 ng/ml) for 24 h were stimulated with rhGH (500 ng/ml) for 12 h. DRB (72 µM) or vehicle was added to the cells, and total RNA was isolated at 0, 30, 60, 90, and 120 min following the addition of DRB by using the Qiagen RNeasy kit per the manufacturer’s instructions. Quantitative real-time PCR was performed by reverse transcribing 5 µg of RNA using oligo(dT), random primers, and Superscript III (Invitrogen) to produce cDNA at a total volume of 20 µl. Real-time PCR was performed by using β-actin as endogenous control and an ABI 7900HT Real-Time PCR system (Applied Biosystems, Foster City, CA).

The relative abundance of IGF-I, Spi 2.1, PRLr mRNA, and actin mRNA were determined by RT-PCR, normalized to actin mRNA, and were reported as fold induction.

**Statistical methods.** Data are presented as means ± SE and represent the results of at least three independent experiments. The Northern blot and immunoblot data are expressed as relative densitometry units. Statistical evaluation of the data was performed by ANOVA followed by the Tukey-Kramer multiple-comparison test with the use of Instat GraphPad 5.02 (San Diego, CA). Differences among means were considered significant at \( P < 0.05 \).

---

**Fig. 4. GH-induced JAK2 phosphorylation in IL-6-treated cells.** Cells were treated with 10 ng/ml IL-6 for 24 h and then stimulated with 500 ng/ml rhGH for 5, 15, 30, 60, and 90 min. A: cell lysate immunoblotted with anti-JAK2 polyclonal antibody. B: lysate immunoblotted with anti-phospho-JAK2 polyclonal antibody. C: densitometry data for phosphorylated JAK2 were normalized to total protein, presented as RDU, and expressed as means ± SE. Blots are representative of experiments done at least 6 times.
RESULTS

IL-6 inhibits the induction of IGF-I and Spi 2.1 mRNA by GH. To investigate the role of IL-6 in hepatic GH resistance, we initially examined the effects of IL-6 on the GH-inducible genes IGF-I and Spi 2.1 by Northern blot. As shown in Fig. 1, incubation of hepatocytes with rhGH resulted in a greater than threefold increase in the abundance of IGF-I mRNA (\(P < 0.001\) vs. control). Treatment of CWSV-1 cells with IL-6 alone had no effect on basal IGF-I expression. Interestingly, the effects of IL-6 on the regulation of IGF-I by GH appear to be time dependent because incubation of hepatocytes with IL-6 for 4 h did not significantly influence the induction of IGF-I by GH (Fig. 1A; 5% reduction in mRNA after 4 h of IL-6), whereas preincubation with IL-6 for 12 h (Fig. 1B; 28% inhibition; \(P < 0.01\) vs. GH) to 24 h (Fig. 1C; 30% inhibition; \(P < 0.01\) vs. GH) significantly inhibited the induction of IGF-I mRNA following GH administration. Similar results were seen when examining the effect of IL-6 on GH-induced Spi 2.1 expression (Fig. 2). CWSV-1 cells exhibited a threefold increase in expression of Spi 2.1 mRNA after treatment with GH (Fig. 2A, \(P < 0.05\) vs. control; Fig. 2B, \(P < 0.001\) vs. control). Treatment with IL-6 alone had no effect on basal Spi 2.1 mRNA expression. Pretreatment with IL-6 for 4 h did not greatly affect induction of Spi 2.1 by GH (Fig. 2A); however, pretreatment with IL-6 for 24 h significantly reduced GH stimulation of Spi 2.1 mRNA expression (Fig. 2B; 47% inhibition; \(P < 0.001\) vs. GH).

Initiation of GH signaling in IL-6-treated hepatocytes. To determine whether IL-6 influenced the time course or magnitude of GH signaling, we examined the effects of IL-6 incubation on total GHR levels and the time course of GHR phosphorylation in GH-stimulated cells (Fig. 3). Neither preincubation of hepatocytes with IL-6 nor stimulation with GH significantly altered total GHR protein relative to baseline. As shown in Fig. 3B, a relatively low level of tyrosine-phosphorylated GHR was observed under basal conditions. Following the addition of rhGH, levels of tyrosine-phosphorylated GHR increased 2.5-fold at 5 min after treatment and subsequently decreased to basal levels by 60 min after GH stimulation. The time course and magnitude of GHR phosphorylation were not significantly altered by IL-6 pretreatment (Fig. 3C).

The tyrosine phosphorylation of JAK2 by the activated GH-GHR2 complex represents a critical step in the initiation of GH signaling (30, 31, 65). Therefore, we measured total JAK2 protein and the time course of JAK2 phosphorylation following GH stimulation in CWSV-1 hepatocytes (in the presence or absence of IL-6). Figure 4A shows that neither IL-6 nor GH influenced the relative abundance of total JAK2 protein in the hepatocytes. Stimulation of the cells with GH increased tyrosine-phosphorylated JAK2 above baseline at 5 min, which maintained through the 90-min time course of treatment (Fig. 4B). IL-6-treated cells showed no significant difference in either the time course or the level of tyrosine-phosphorylated JAK2 following GH stimulation (Fig. 4B). Consequently, IL-6 does not appear to influence the initiation of GH signaling by altering total or tyrosine-phosphorylated JAK2.

Propagation of GH signaling via the JAK/STAT pathway. To determine whether IL-6 inhibits the propagation of GH signaling via the STAT5 pathway, we examined the time course of STAT5b phosphorylation and DNA binding following GH stimulation in CWSV-1 cells. Cells were treated with 10 ng/ml IL-6 for 24 h and then stimulated with 500 ng/ml rhGH for 5, 30, 60, 90, and 120 min, \(n = 6\). A: cell lysate immunoblotted with anti-STAT5b polyclonal antibody. B: lysate immunoprecipitated with anti-STAT5b and immunoblotted with PY20 antibody, representative of experiments performed at least 6 times. C: densitometry data for phosphorylated STAT5b were normalized to total protein, presented as RDU, and expressed as means ± SE. \(P < 0.05\) vs. GH at 90 min.
stimulation in CWSV-1 hepatocytes (in the presence and absence of IL-6). First, the relative abundance of STAT5b protein was measured in cell lysates harvested over time following stimulation with GH. Total STAT5b protein levels were not altered in cell lysates harvested 0 to 120 min following GH stimulation (with or without IL-6; Fig. 5A). Before GH stimulation, tyrosine-phosphorylated STAT5b was barely detected in either control or IL-6-treated cells (Fig. 5B). Following the addition of GH, the relative abundance of tyrosine-phosphorylated STAT5b protein in the lysates increased approximately fivefold from 0 to 5 min, then decreased over time. Pretreatment with IL-6 tended to impair the ability of GH to increase STAT5 phosphorylation, especially between 60 and 120 min. However, only the difference at 90 min (38% reduction) achieved statistical significance compared with time-matched control hepatocytes. To determine the effects of IL-6 on functional activity of phosphorylated STAT5 following translocation to the nucleus, we accessed the DNA-binding activity of nuclear protein extracts by using an EMSA to a STAT5 β-casein promoter sequence (52, 64). The STAT5 DNA-binding activity of nuclear protein was increased at both 15 and 60 min after GH treatment of the cells (Fig. 6). DNA binding by STAT5 was increased 14-fold (vs. baseline) at 15 min after GH treatment (Fig. 6B; \( ^*P < 0.001 \) vs. untreated cells at 0 min). This binding activity was diminished but still evident at 60 min (\( ^*P < 0.001 \) vs. untreated cells at 0 min). Pretreatment of the cells with IL-6 diminished STAT5 DNA-binding activity at both 15 min (\( ^*P < 0.01 \) vs. GH at 15 min) and 60 min (\( ^*P < 0.001 \) vs. GH at 60 min) following GH stimulation compared with GH treatment alone. The specificity of the STAT5 binding reaction was confirmed by the ability of antibody specific for STAT5b to shift the mobility of the STAT5 DNA complex (Fig. 6A, lane 7). The specificity of STAT5 binding conditions in this EMSA was previously demonstrated by using cold consensus and mutated competitor sequences as described by Yumet et al. (63, 64). These observations indicate that IL-6 alters the ability of activated STAT5 to bind to DNA.

Propagation of GH signaling via the MAP kinase pathway. GH also uses the MAP kinase pathway to regulate inflammatory and metabolic responses (45, 51, 52). Serine phosphorylation of STAT5 via the MAP kinase pathway regulates STAT5-mediated gene transcription. The potential for IL-6 to

![Fig. 6. Effects of IL-6 on GH-induced STAT5 DNA-binding activity. CWSV-1 cells were treated with 10 ng/ml IL-6 for 24 h and then stimulated with 500 ng/ml rhGH for 15 and 60 min. A: nuclear extracts (5 μg) were used in an EMSA with a STAT5-labeled probe as described in METHODS. P, probe alone, C, untreated cells. In lane 7, protein from GH after 15 min was used in the presence of anti-STAT5b antibody. The migration of the shifted DNA-protein complex and supershift complex are indicated. The blot is representative of experiments done 6 times. B: densitometry data for the STAT5-DNA complex are expressed as means ± SE. \( ^*P < 0.001 \) vs. untreated cells; \( ^{+}P < 0.01 \) vs. GH at 15 min; \( ^{+}P < 0.001 \) vs. GH at 60 min.]
alter GH-mediated activation of the MAP kinase cascade was evaluated by measuring total and phosphorylated ERK1 and ERK2 in cells harvested 0–60 min after GH stimulation (52). Neither GH stimulation nor IL-6 pretreatment altered total ERK1 or ERK2 levels relative to control values (Fig. 7A). As shown in Fig. 7B, low levels of phosphorylated ERK1 and ERK2 were detected in control cells (in the presence or absence of IL-6). Phosphorylated ERK1/2 increased twofold from 0 to 5 min following GH stimulation and returned to baseline by 60 min after GH treatment (Fig. 7B). IL-6 did not alter GH-mediated phosphorylation of ERK1 and ERK2 at any time point compared with GH treatment alone (Fig. 7C). Consequently, IL-6 does not appear to influence the amplitude or duration of activated ERK1 and ERK2 in the CWSV-1 hepatocytes.

**Effect of IL-6 on Spi 2.1 and PRLr promoter activity.**

Transient transfections in CWSV-1 cells were used to determine the effects of pretreatment with IL-6 and GH stimulation on Spi 2.1 promoter activity. Cells were transfected with the Spi 2.1 promoter luciferase construct. The hepatocytes were treated with IL-6 for 4 or 24 h followed by GH stimulation for 4 h. As shown in Fig. 8, IL-6 alone did not alter basal Spi 2.1 promoter activity. In contrast, GH stimulation significantly stimulated Spi 2.1 promoter activity (*P < 0.001 vs. control*). The inhibitory effect of IL-6 on GH induction of Spi 2.1 promoter activity appeared to be time dependent. As shown in Fig. 8A, pretreatment with IL-6 for 4 h before GH stimulation did not alter GH stimulation of the Spi 2.1 promoter, whereas an 80% inhibition was observed after pretreatment with IL-6 for 24 h (Fig. 8B; *bP < 0.001 vs. GH alone*), indicating a time-dependent mechanism of inhibitory activity.

The experiment was repeated with the use of another GH-inducible promoter construct to determine whether these effects were specific to Spi 2.1 or were a more generalized effect on GH-inducible promoter activity. CWSV-1 cells underwent transient transfection with the PRLr promoter luciferase construct. At 18 h after transfection, cells were treated in the presence or absence of IL-6 (10 ng/ml) for 24 h, followed by GH stimulation (500 ng/ml) for 12 h, and luciferase activity was examined. As shown in Fig. 9, IL-6 treatment alone did not affect basal PRLr promoter activity. GH treatment caused a fourfold increase in promoter activity (*P < 0.001 vs. control*).
control), whereas pretreatment with IL-6 resulted in a 42% decrease in GH-induced PRLr promoter activity ($P < 0.001$ vs. GH alone).

**Effect of IL-6 on GH-inducible mRNA stability.** The effect of IL-6 on IGF-I, Spi 2.1, and PRLr mRNA stability was examined to determine whether cytokine pretreatment increased the degradation of GH-inducible mRNA transcripts. The half-life of the IGF-I, Spi 2.1, and PRLr mRNA species was the same in the DRB+GH and IL-6+GH+DRB groups. Collectively, these results suggest that IL-6 does not significantly alter GH-inducible mRNA stability in CWSV-1 hepatocytes.

**DISCUSSION**

The development of hepatic GH resistance is a major metabolic derangement in patients with catabolic illness including sepsis, trauma, burns, and inflammatory bowel disease (2–4, 9–13, 29, 60). Transient elevations in TNF or IL-1 are seen during systemic inflammation and contribute to the development of hepatic GH resistance (20–22, 33, 52, 64). However, both of these cytokines secondarily stimulate hepatic synthesis and secretion of IL-6, a pleiotropic inflammatory cytokine that is elevated in plasma for a prolonged time period in patients with catabolic illness (11, 22, 26). Elevations in plasma IL-6 are associated with reductions in circulating IGF-I and impaired growth in experimental colitis. More recently, the −174 IL-6 G/C promoter polymorphism, which results in increased IL-6 transcription, was associated with elevated C-reactive protein and impaired growth in children with Crohn’s disease (50). In the current study, we describe the effects of IL-6 on GH-inducible gene expression and GH signaling in CWSV-1 hepatocytes.

CWSV-1 is an SV40 transformed cell line derived from normal rat hepatocytes. CWSV-1 cells demonstrate many characteristics that make them ideal for regulatory studies of liver-specific proteins. First, the regulation of albumin synthesis and secretion in CWSV-1 is similar to that observed in normal liver (32, 61). Second, in contrast to primary hepatocytes, CWSV-1 cells do not require frequent isolation because they replicate in vitro and do not have to be grown on a matrix (36). Third, CWSV-1 cells express GH receptor and synthesize IGF-I in response to GH (1, 32, 52, 64). Fourth, the regulation of GH signaling in this cell line is well characterized (1, 46–48, 52). CWSV-1 cells incubated with IL-6 for 4 h demonstrate a normal twofold induction of IGF-I following GH stimulation (Fig. 1). However, preincubation with IL-6 for 12–24 h significantly inhibits the induction of IGF-I by GH. The effects of IL-6 on Spi 2.1 expression were examined to determine whether the inhibitory effects of IL-6 were unique to IGF-I or a more general regulatory phenomenon affecting other GH-inducible genes. Spi 2.1 is a GH-inducible negative acute-phase protein that inhibits neutrophil-mediated lung injury and has been used by others to study GH-inducible gene expression (5–7, 34, 53). CWSV-1 cells treated with IL-6 for 4 h demonstrate a 2.5-fold induction of Spi 2.1 by GH, whereas incubation with IL-6 for 24 h significantly inhibits the GH-induced increase of Spi 2.1 (Fig. 2). The cis-regulatory regions for both Spi 2.1 and IGF-I contain tandem STAT5 elements, which are important in regulating GH-inducible gene expression (5, 6, 34, 58, 59). Although the induction of Spi 2.1 by GH was inhibited by IL-6 in primary hepatocytes (6), this is the first study to demonstrate an inhibitory effect of IL-6 on GH-inducible IGF-I expression.

Hepatic GH resistance may be caused by reductions in hepatic GHR levels or by postreceptor defects in GH signaling and gene expression. Reductions in GHR expression or the binding of GH to its cognate receptor have been identified in liver from animals with GH resistance (16, 18, 19). A 45% reduction in GHR mRNA and protein were identified in liver tissues from IL-10 knockout mice with colitis and hepatic GH resistance (19). Defalque et al. (16) identified a 50% reduction in hepatic GH binding sites 5–10 h after LPS administration. Furthermore, transgenic mice that overexpress IL-6 have a decreased GHR mRNA content in liver relative to controls (37). Despite the reductions in hepatic GHR identified in these studies, the relative abundance of GHR protein in liver from LPS-treated or intra-abdominal sepsis animals is unaltered when measured by immunoblot analysis (40, 63). The relative abundance of GHR and GH signaling proteins (JAK2, STAT5, ERK1/2) was not influenced by pretreatment with IL-6 in the current study and therefore is an unlikely mediator of the inhibitory effects of IL-6 on GH-inducible gene expression.

Postreceptor defects in GH signaling via the JAK2/STAT5 pathway have also been identified in the pathogenesis of hepatic GH resistance (1, 40, 52, 63, 64). The duration of STAT5 phosphorylation (following GH stimulation) is decreased in liver from LPS-treated or septic rats compared with control values (40, 63). Decreased STAT5 phosphorylation and associated reductions in GH-inducible IGF-I expression are also observed in CWSV-1 cell lines in response to TNF (64). The current study is the first to examine the effects of IL-6 on the time course of GH signaling by both the JAK/STAT and MAP kinase pathways. Neither the magnitude nor the duration of GHR, JAK2, or ERK1/2 phosphorylation were significantly decreased in hepatocytes treated with IL-6 before GH stimulation. Although the ratio of phosphorylated to total STAT5 was decreased at the 90-min time point in the IL-6+GH group (Fig. 5), it was similar to GH alone for the 5-min, 30-min,
IL-6 inhibits GH gene expression

The inhibitory effect of TNF on GH-inducible gene expression appears to be independent of STAT5 activity (1). Therefore, we examined the effects of IL-6 on GH-inducible STAT5 activity (by EMSA) and promoter activity (using Spi 2.1 and PRLr promoter reporter constructs). Nuclear protein from IL-6-treated cells demonstrates decreased binding to STAT5 GAS sequences 15 and 60 min after GH stimulation (Fig. 6). When coupled with the inhibitory effects of IL-6 on GH-inducible Spi 2.1 (Fig. 8) and PRLr (Fig. 9) promoter activity, our results suggest that IL-6 inhibits GH-inducible promoter activity.

To eliminate the possibility that IL-6 also enhances the degradation of GH-inducible mRNA transcripts, we examined the effects of IL-6 on the time course of IGF-I mRNA degradation in DRB-treated cells. There was no evidence that mRNA stability of GH-inducible genes like IGF-I or PRLr was significantly decreased by IL-6. Thus the inhibitory effect of IL-6 on GH-inducible promoter activity appears to represent the predominant mechanism for IL-6-mediated GH resistance. The cis-regulatory regions of the Spi 2.1, PRLr, and IGF-I genes all contain STAT5 GAS sequences, which have been implicated in the regulation of gene expression by GH (23, 34, 53, 58, 59). Although the time course of STAT5 DNA binding is decreased in IL-6-treated cells following GH stimulation, the current studies are unable to ascertain whether these changes are correlative or causative.

The rat IGF-I gene, as in the human, comprises over 80 kb and is composed of six exons and five introns (39). IGF-I gene expression is regulated by transcription initiation at two separate 5′ promoter regions (P1 and P2), alternative mRNA splicing, and 3′ mRNA polyadenylation (39). The 5′ IGF-I promoter region from −1500 to +300 is required for basal IGF-I promoter activity (41). However, the regulatory sequences responsible for GH-inducible IGF-I expression have only recently been described (56). An 84-bp sequence called HS7 serves as a cis-regulatory region for GH-inducible IGF-I expression and was recently localized to intron 2 of the rat IGF-I gene (56). HS7 contains tandem STAT5 binding sites and a DNase1-hypersensitive site (56). A second cis-regulatory region (352 bp in size) with tandem STAT5 sites was identified −75 kb 5′ to the IGF-I gene in Hep G2 cells (57). Several other GH-inducible genes including Spi 2.1 and acid-labile subunit also contain tandem STAT5 binding sites and a DNase1-hypersensitive site in their cis-regulatory region (5, 7, 23, 51). STAT5 DNA binding clearly plays a critical role in activating GH-inducible gene expression (27, 38, 44, 58, 59). However, the correlation of GH-inducible gene transcription with DNase1 sensitivity and the acetylation of core histones with STAT5 binding suggests that interactions between STAT5b, other transcription factors, and transcriptional coactivators/corepressors are also important in regulating GH-inducible gene expression (53, 58, 59, 62). The transcriptional factors involved in regulating GH-inducible IGF-I expression remain poorly defined. However, interactions between STAT5b and other transcription factors, including the glucocorticoid receptor, hepatocyte nuclear factor-4, peroxisome proliferator-activated receptors, and CCAT/enhancer-binding protein-B have been shown to regulate STAT5s-inducible gene expression (27, 38, 44). Likewise, interactions between STAT5b and chromatin-modifying transcriptional coactivators/corepressors, including p300/CREB-binding protein, nuclear receptor coactivator 1, and GAGA binding proteins have also been shown to enhance or inhibit STAT5-mediated gene transcription (53, 58, 59, 62). Additional studies will be required to determine the exact mechanisms by which IL-6 inhibits GH-inducible gene expression.

In summary, the inhibitory effects of IL-6 on GH-inducible gene expression are time dependent, requiring 12–24 h to manifest. IL-6 does not alter the relative abundance of GHR or GH-signaling proteins, and neither the magnitude nor the duration of GH signaling was significantly decreased in CWSV-1 hepatocytes treated with IL-6 before GH stimulation. The inhibitory effects of IL-6 on GH-inducible promoter activity affect multiple GH-inducible genes (Spi 2.1, IGF-I, and PRLr) and are associated with reductions in STAT5 DNA binding. Collectively, these results implicate postreceptor defects in GH-inducible gene expression in the pathogenesis of IL-6-mediated hepatic GH resistance.

Acknowledgments

We thank H. C. Isom (Department of Microbiology, Pennsylvania State College of Medicine) for providing the CWSV-1 cells.

Grants

This work was supported in part by National Institute of General Medicine Grants GM-55639 (to R. N. Cooney), GM-38032 (to C. H. Lang), and T32-GM-64332 (to T. A. Ahmed and M. D. Buzzelli).

References

G1802 IL-6 INHIBITS GH GENE EXPRESSION


