Tumor necrosis factor mediates hepatic growth hormone resistance during sepsis

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Yumet, Gladys, Margaret L. Shumate, Patrick Bryant, Cheng-Mao Lin, Charles H. Lang, and Robert N. Cooney. Tumor necrosis factor mediates hepatic growth hormone resistance during sepsis. Am J Physiol Endocrinol Metab 283:E472–E481, 2002; 10.1152/ajpendo.00107.2002.—During sepsis, growth hormone (GH) resistance contributes to the catabolism of muscle protein. To determine the role of tumor necrosis factor (TNF) as a mediator of GH resistance, we examined the effects of a TNF antagonist [TNF-binding protein (TNFbp)] on the GH/insulin-like growth factor (IGF) I system during abdominal sepsis. To investigate potential mechanisms, the effects of TNF on the IGF-I response to GH and GH signaling were examined in cultured rat hepatocytes (CWSV-1). Three groups of rats were studied: Control, Sepsis, and Sepsis + TNFbp. Liver, gastrocnemius, and plasma were collected on day 5. In gastrocnemius, neither sepsis nor TNFbp altered the abundance of IGF-I mRNA. However, septic rats demonstrated an increase in circulating GH and a reduction in plasma IGF-I concentrations that was ameliorated by pretreatment with TNFbp. Liver from septic rats demonstrated a 50% reduction in GH receptor (GHR) and IGF-I mRNA on day 5 that was attenuated by TNFbp. However, the abundance of GHR protein was not different in liver from Control, Sepsis, or Sepsis + TNFbp rats. Consequently, a decreased amount of hepatic GHR does not explain the GH-resistant septic state. In CWSV-1 hepatocytes, TNF-α had no effect on GHR protein level but inhibited the induction of IGF-I mRNA by GH. Nuclear protein from TNF-treated hepatocytes demonstrated similar levels of phosphorylated signal transducer and activator of transcription-5 (STAT5) and DNA binding relative to controls 5 min after GH treatment. However, both of these parameters were decreased (vs. control) in TNF-treated cells 60 min after GH treatment. Collectively, these results suggest that TNF mediates hepatic GH resistance during sepsis by inhibiting the duration of signaling via the janus kinase-2/STAT5 pathway.

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TNF inhibits GH signaling

vate the transcription of GH target genes like serine protease inhibitor 2.1 (23). Although GH stimulates the synthesis and secretion of IGF-I by liver, the exact mechanisms by which GH stimulates IGF-I transcription are still unresolved. However, studies in STAT5b knockout mice suggest that STAT5 is required for both basal and GH-induced expression of hepatic IGF-I (14). Sepsis also appears to impair hepatic GH action, as evidenced by a smaller GH-induced increase in plasma IGF-I in septic patients compared with control subjects (13).

We hypothesized that TNF-α mediates the development of “GH resistance” during abdominal sepsis by inhibiting GH signaling. To test this hypothesis, we examined the effects of TNF-binding protein (TNFbp), a potent TNF antagonist (17), on the expression of GHR and IGF-I in muscle and liver from septic rats. The results suggest that TNF is an important mediator of GH resistance in liver and acts by inhibiting the duration of GH signaling via the JAK/STAT pathway.

**EXPERIMENTAL PROCEDURES**

**Materials.** Recombinant human GH (rhGH, Pharmacia and Upjohn, Stockholm, Sweden) was used in all experiments. The rat TNF-α was obtained from R & D Systems (Minneapolis, MN). TNFbp, a 555-soluble TNF receptor, was a kind gift of Carl Edwards (Amgen, Thousand Oaks, CA). Plasmid containing the rat IGF-I cDNA was a kind gift from Peter Rotwein (Oregon Health Science) (47), and the rat GR DNA was from L.S. Mathews (University of Michigan) (39). Antibodies included polyclonal GHR antibody, a kind gift from W. R. Baumbach (American Cyanamid, Princeton, NJ), used at a dilution of 1:250 (46), a rabbit polyclonal STAT5b antibody raised against a peptide mapping the carboxy terminus (sc-835, Santa Cruz Biotechnology, CA), and PY20 phosphotyrosine antibody conjugated with horseradish peroxidase (HPR) from BD Transduction Laboratories (San Diego, CA).

**Animals.** Three groups of male Sprague-Dawley rats (200–250 g; Charles River Breeding Laboratories, Wilmington, MA) were studied: Control, Sepsis, and Sepsis + TNFbp. A Control + TNFbp group was not included in all studies, because preliminary results suggested that TNFbp had no effect on the GH/IGF-I system in control animals (Table 1). Saline (1.0 ml) or TNFbp (1 mg/kg, 1.0 ml) was injected subcutaneously daily with the initial dose administered 4 h before induction of sepsis. The dose and timing of TNFbp administration were based on previous work demonstrating inhibitory plasma levels and biological activity in the model of abdominal sepsis used in the current study (11).

Animals were anesthetized by intramuscular injection of ketamine (80 mg/kg) and acepromazine (1 mg/kg). A midline abdominal incision was made, the intestines were briefly manipulated, and the incision was closed in Control rats. Chronic abdominal sepsis was created by implantation of a fecal-agar pellet (1.5 ml) inoculated with 104 colony-forming units of Escherichia coli and 105 colony-forming units of Bacteroides fragilis in the abdominal cavity of the Sepsis and Sepsis + TNFbp groups (9). After recovery from surgery, animals were housed individually. Food intake and body weight were monitored daily and were not significantly influenced by TNFbp, as previously described by our laboratory (9).

On day 5, rats were anesthetized as described and liver and gastrocnemius were excised. Tissues were frozen in liquid nitrogen by use of precooled clamps and stored at −70°C until RNA and protein isolation was performed. Blood was obtained by aortic puncture into heparinized syringes and plasma was stored at −20°C until analyzed. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University, College of Medicine.

**Cell culture experiments.** CWSV-1 cells were obtained from Dr. Harriet Isom (Dept. of Microbiology, College of Medicine, Pennsylvania State University). CWSV-1 is an SV40 transformed rat hepatocyte cell line that has been extensively characterized and demonstrates many of the important regulatory mechanisms observed in normal liver tissue (32, 37, 55). CWSV-1 cells were grown in RPCD medium (32, 36, 53) for 48 h. TNF-treated cells were incubated with 10 ng/ml TNF-α for 4 h, and then 500 ng/ml rhGH were added for the indicated time periods.

**Northern blot analysis.** The relative abundance of IGF-I and GH mRNA was determined by Northern blot analysis (51). Total RNA was isolated from liver tissue using the TOTally RNA Isolation Kit (Ambion, Austin, TX). CWSV-1 hepatocyte RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). Twenty micrograms of total RNA were separated on a denaturing agarose-formaldehyde gel and transferred onto a GeneScreen hybridization transfer membrane (NEN Life Science Products, Boston, MA) following standard Northern blot techniques. cDNA probes were labeled using [32P]dCTP and the random primer technique (Multiprime DNA Labeling System, Amersham Pharmacia Biotech, Piscataway, NJ) and purified by gel filtration chromatography on G-25 Sephadex Quick Spin columns (TE; Roche Diagnostics, Indianapolis, IN). 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 utilized as a probe (47). The rat IGF-I gene exhibits alternate splicing in the first exon, resulting in the production of multiple exon 1-derived transcripts. The liver expresses all of the exon 1-derived IGF-I mRNA species ranging in size from 7.5 to 0.8 kb, with the high-molecular-weight species of 7.5 kb representing the majority (80%) of the IGF-I mRNA present. In the muscle, the 7.5-kb IGF-I transcript represents almost 100% of IGF-I mRNA (1). For the GHR, a 600-bp HincII-HindIII fragment corresponding to the rat GHR-I cDNA containing exons 1, 3, 4, 5, and 6 was used as a probe (39). Membranes were prehybridized in 0.5 M sodium phosphate, pH 7.2, with 1% powdered milk, 1 mM EDTA, pH 8.0, 2% SDS, 2X Denhardt’s solution, and 200 μg/ml fish sperm DNA. Prehybridization was conducted for 1 h at 65°C. Hybridization was conducted overnight at 65°C with radiolabeled cDNA probes. After a washing, the blots were exposed to x-ray film and the bands were quantitated using a densitometer (Fuji, New York, NY). The results were similar to those obtained for the liver and will not be presented here.

**Table 1. Effects of TNFbp on sepsis-induced changes in plasma hormone concentrations**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Plasma IGF-I</th>
<th>Plasma GH</th>
</tr>
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<tbody>
<tr>
<td>Control (8)</td>
<td>1085 ± 32</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>Control + TNFbp (9)</td>
<td>1298 ± 14</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Sepsis (12)</td>
<td>781 ± 12*</td>
<td>32 ± 3*</td>
</tr>
<tr>
<td>Sepsis + TNFbp (7)</td>
<td>1063 ± 15</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SE in ng/ml; no. of animals are given in parentheses. TNFbp, tumor necrosis factor-binding protein; IGF-I, insulin-like growth factor I; GH, growth hormone. *P < 0.05 vs. Control, Control + TNFbp and Sepsis + TNFbp.
at −70°C for 2−5 days to Kodak X-Omat AR film in a cassette equipped with a Du Pont Lightning Plus intensifying screen. The exposed autoradiographs were scanned using a densitometer (model 100A; Molecular Dynamics, Sunnyvale, CA). Band intensities were determined using the Quantity One version 2 software (Bio-Rad Laboratories, Hercules, CA). Northern blots were stripped and reprobed with the 18S ribosomal subunit message to confirm the absence of variations in RNA loading. An oligonucleotide specific for 18S was 5′-end labeled using T4 polynucleotide kinase (Promega, Madison, WI) 5′-GTATGTGCTATCTGGGTT-3′ and used as a probe. Data are reported as relative densitometry units after normalization to 18S rRNA message.

Preparation of liver membrane extracts. Hepatic membranes were isolated using a modification of Posner et al. (27). Briefly, powdered frozen liver was homogenized in 10 vol of ice-cold 0.3 M sucrose, pH 7.4, by use of a Brinkman polytron type PT-10 at a setting of 5 for 25 s (43). The homogenate was centrifuged for 15 min at 600 g at 4°C. Individual pellets were resuspended in cold 0.5 M sucrose buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 40,000 g twice for 45 min. The pellets (membrane fraction) were gently homogenized in 0.3 M sucrose, 25 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, and leupeptin using a 25-gauge needle and 1-ml syringe assembly.

Isolation of whole cell lysates and nuclear protein. Cells grown in culture dishes were 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 mM EDTA, 60 mM KCl, 0.5% IGEPAL CA-630, 1 mM dithiothreitol (DTT), and 1 mM sodium orthovanadate) was added directly to the plates and incubated for 30 min. Lysates were collected and cleared by centrifugation at 7,000 g for 5 min. Nuclear extracts from CWSV-1 cells were prepared as previously described (48). Briefly, cells were washed three times with cold PBS and then scraped in PBS containing 0.5 mM PMSF. Each cell pellet was washed and resuspended in lysis buffer: 10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl, 0.5% IGEPAL CA-630, 1 mM dithiothreitol (DTT), and 1 mM PMSF. Lysed cells were spun at 1,200 g for 5 min, and nuclei were washed in lysis buffer without detergent. Nuclei were resuspended in buffer containing 250 Tris, pH 7.8, 60 mM KCl, 1 mM DTT, and 1 mM PMSF and then subjected to three cycles of freezing and thawing. Lysed nuclei were spun for 15 min at 7,000 g, and the supernatant was collected and quickly frozen in liquid nitrogen and stored at −70°C.

Western blot analysis and immunoprecipitation. Equal amounts of protein were electrophoresed on a 7.5 or 4−15% gradient-resolving polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore, Bedford, MA), by means of standard electrophoretic procedures. Membranes were blocked for 1 h at room temperature with 5% nonfat milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) for GHR detection. For phosphotyrosine detection of STAT5 and GHR, membranes were blocked overnight at 4°C in 5% BSA in TBS-T (TBS with 0.1% Tween-20) and, for GHR and STAT5 detection, 5% milk in TBS-T.

The membranes were incubated overnight at room temperature with a polyclonal antibody specific for rat GHR (46) or incubated for 1 h at room temperature with a polyclonal antibody against STAT5b. The secondary antibody utilized was linked to horseradish peroxidase (Accurate Chemical and Scientific, Westbury, NY). The wash step was repeated, and the antibodies were visualized using ECL-Plus (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Densitometry scans of the exposed films were performed using a densitometer, and intensity was analyzed using Quantity One version 2 software (PDI, Bio-Rad).

Cell lysates (100−500 µg) were immunoprecipitated in HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.2 mM Na3VO4, 0.2 mM PMSF, 2 µg/ml aprotinin), 2 µg of STAT5b antibody, or GHR antibody and 20 µl of agarose-conjugated beads (protein A-agarose; Santa Cruz Biotechnology) overnight at 4°C. Beads were collected and washed three times, and 2× sample Laemmli buffer (Bio-Rad) was added to samples and boiled for 5 min. Immunocomplexes were resolved using SDS-PAGE, and Western blot analysis was performed as described.

Electrophoretic mobility shift assay. Oligonucleotides for gel shift assays were as follows: complementary strands to the rat β-casein promoter 5′-GGA CTT CTT GGA ATT AAG GGA-3′ were labeled independently using T4 kinase (Promega) and [γ-32P]ATP, annealed, and purified on an 18% PAGE (5). STAT5 consensus and mutant oligonucleotides were used as cold competitors (Santa Cruz Biotechnology). Nuclear protein from CWSV-1 cells (3−5 µg) was used in a binding reaction containing 2 µg of poly(dI-dC), 0.5−1.0 ng of probe (50,000 cpm), and 1× binding buffer (10 mM Tris, pH 7.5, 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl) (44). Reactions were incubated for 30 min at 25°C and then electrophoresed on a prerun 4% PAGE/0.5× TBE for 2.5 h at 25 mA at 4°C. To supershift complexes, STAT5b antibody (sc-835-X, Santa Cruz Biotechnology) was added to the reactions and incubated for 30 min at 25°C before the addition of the probe. Gels were dried onto a Whatman paper with a gel dryer (Bio-Rad) and exposed to film.

GH and IGF-I determination. IGF-I concentrations in plasma were determined by radioimmunoassay (RIA), as previously described (34) with the use of an antibody supplied by the National Hormone and Pituitary Program (Rockville, MD). Before the assay was performed, plasma was extracted using an acid-ethanol solution and subsequent cryoprecipitation to remove binding proteins. The eluate was evaporated, and the dried sample was reconstituted with phosphate buffer for IGF-I determination. GH concentrations in plasma were determined using commercially available RIA with a rat GH125I assay system (Amersham Pharmacia Biotech).

Statistical analysis. Data are expressed as means ± SE for 7−12 animals in each group. Cell culture data 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 analyzed by analysis of variance (ANOVA) followed by the Tukey-Kramer Multiple Comparison Test or by Student’s t-test (two tailed) using Instat GraphPad 5.02 (San Diego, CA). Differences among means were considered significant at P < 0.05.

RESULTS

Role of TNF in mediating sepsis-induced changes in GH/IGF-I axis in vivo. The effects of TNFbp and the septic insult on the GH/IGF-I axis were assessed by evaluating plasma levels of GH and IGF-I (Table 1). Septic rats demonstrated a twofold increase in circulating GH on day 5 of chronic abdominal sepsis (P < 0.05 vs. Control and Sepsis + TNFbp). Despite the increase in circulating GH observed in septic rats, plasma IGF-I levels were decreased by ~30% (P < 0.05

AJP-Endocrinol Metab • VOL 283 • SEPTEMBER 2002 • www.ajpendo.org
vs. Control). Although treatment with TNFbp did not influence either GH or IGF-I in control animals, it significantly attenuated the sepsis-induced changes in plasma GH and IGF-I levels. To determine whether the effect of sepsis on the GH/IGF-I axis was seen in both liver and muscle, we examined the expression of IGF-I in both tissues. In gastrocnemius, neither sepsis nor the administration of TNFbp (Fig. 1A) altered the relative abundance of IGF-I mRNA. In contrast, IGF-I mRNA content in liver from septic rats was decreased on day 5 compared with Control values, and pretreatment with TNFbp prevented the sepsis-induced decrease in hepatic IGF-I mRNA (Fig. 1, B and C).

The expression of GHR mRNA and protein in liver tissue was examined in the different experimental groups to determine whether decreased expression of GHR could be responsible for the reductions in hepatic IGF-I mRNA observed in septic rats. As shown in Fig. 2, A and B, sepsis produced a 50% reduction in hepatic GHR mRNA. The sepsis-induced decrease in GHR mRNA observed in liver was ameliorated by the administration of TNFbp. Despite the reduction in hepatic GHR mRNA observed in septic rats, the relative abundance of hepatic GHR protein was not significantly decreased in hepatic membrane preparations from septic rats with or without TNFbp administration compared with samples from control rats (Fig. 2C).

Effects of TNF-α on GH-induced changes in IGF-I under in vitro conditions. To further investigate the complex events that occur in vivo during the septic insult and to dissect the role of individual cytokines, we examined the effects of TNF-α on IGF-I synthesis by CWSV-1 hepatocytes. Of importance, CWSV-1 hepatocytes synthesize IGF-I mRNA in response to GH, and GH signaling in this cell line has been extensively characterized (23, 32, 45). The time course of IGF-I mRNA expression after GH stimulation was determined using 500 ng/ml GH (Fig. 3A). IGF-I mRNA levels were not significantly altered at 1 h after GH stimulation. However, a twofold increase in IGF-I mRNA was observed at 24 h, and a fourfold increase in IGF-I mRNA was noted in CWSV-1 cells after 48 h of incubation with 500 ng/ml rhGH (Fig. 3A). As shown in Fig. 3B, incubation of hepatocytes with GH for 18 h resulted in a twofold increase in the abundance of IGF-I mRNA (P < 0.05 vs. Control). Although preincu-
bation of hepatocytes with TNF-α for 4 h had no effect on basal IGF-I expression, it prevented the normal induction of IGF-I mRNA observed after GH administration \( (P < 0.05 \text{ vs. Control} + \text{GH}; \text{Fig. 3C}) \).

Hepatic GHR protein levels were measured in control and TNF-treated CWSV-1 cells before the addition of rhGH. As shown in Fig. 4A, incubation of CWSV-1 hepatocytes with TNF-α for 4 h or with GH for 1 h did not alter GHR protein levels. There was no tyrosine phosphorylation of the GHR under basal conditions or in response to TNF-α. However, phosphorylation of GHR was diminished 60% in cells exposed to TNF-α before addition of GH compared with the increase induced by GH alone at 5 min \( (P < 0.05 \text{ vs. GH at 5 min}; \text{Fig. 4B}) \).

**Activation of the JAK/STAT-signaling pathway represents an important mechanism by which GH induces target gene transcription.** Binding of GH to its membrane-bound receptor stimulates tyrosine phosphorylation of receptor-associated JAK2, the cytoplasmic portion of GHR, and STAT5b in liver (42). Phosphorylated STAT5b translocates to the cell nucleus, where it binds a specific DNA sequence to stimulate target gene transcription. To determine whether TNF-α had an inhibitory effect on the activation of STAT5b by GH, we measured total and phosphorylated STAT5 in cytoplasmic and nuclear extracts from CWSV-1 hepatocytes. Total protein was isolated from cell lysates, and nuclear extracts were harvested 5 and 60 min after stimulation with GH. Total STAT5b protein levels were not altered in cell lysates from cells treated with TNF-α or exposed to GH (Fig. 5A). As shown in Fig. 5B, phosphorylated STAT5b was barely detectable in both control and TNF-treated cells before GH stimulation. However, the abundance of tyrosine-phosphorylated STAT5b protein in the cytoplasmic fraction of control cells was increased more than fourfold at both 5 and 60 min after GH stimulation. The abundance of phosphorylated STAT5b in the cytosolic fraction was also increased more than fourfold in TNF-treated cells exposed to GH for 5 min. However, the phosphorylation of STAT5 was decreased by 40% \( (P < 0.05 \text{ vs. GH at 60 min}) \) in TNF-treated cells after the 60-min incubation with GH (Fig. 5B).

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**Fig. 3.** Expression of IGF-I mRNA in CWSV-1 cells. A: time course of IGF-I mRNA levels after treatment with 500 ng/ml recombinant human (rh)GH at 1, 24, and 48 h. C, untreated cells; +GH, cells treated with GH. B: cells were treated with 10 ng/ml TNF-α for 6 h and then stimulated with 500 ng/ml rhGH (T+GH) for 18 h. Northern blot analysis was performed as described in EXPERIMENTAL PROCEDURES. IGF-I mRNA corresponds to the 7.5-kb exon 1-derived transcript. C: densitometry data for IGF-I mRNA were normalized to 18S rRNA message and expressed as means ± SE. \( *P < 0.05 \text{ vs. Control}; **P < 0.05 \text{ vs. GH}\).

**Fig. 4.** Effect of TNF-α on GHR levels in CWSV-1 cells. Cells were treated with 10 ng/ml TNF-α for 4 h and then stimulated with 500 ng/ml rhGH for 5 and 60 min. A: cytosolic fraction immunoblotted with anti-GHR polyclonal antibody. B: cytosolic fraction immunoprecipitated with anti-GHR and immunoblotted with PY20 (anti-phosphotyrosine) antibody. Blots are representative of experiments done ≥3 times.
Before the addition of GH, only very low levels of total and phosphorylated STAT5 were seen in the nucleus of CWSV-1 hepatocytes (Fig. 5, C and D). Total nuclear STAT5b protein levels were increased about twofold 5 min after GH stimulation in both TNF-α/H9251 and control groups. However, a 30% reduction in total STAT5b was noted 60 min after the addition of GH in TNF-treated cells (P < 0.05 vs. GH at 60 min; Fig. 5C). Similarly, phosphorylated STAT5 levels in the nucleus were increased at 5 and 60 min after GH stimulation. However, the tyrosine-phosphorylated STAT5 was decreased 60 min after GH stimulation in cells pretreated with TNF-α (Fig. 5D).

Phosphorylated STAT5 in the nucleus binds to consensus sequences in the promoter regions of GH-responsive genes, thereby regulating their transcription (27). The DNA-binding activity of STAT5 was measured using an electrophoretic mobility shift assay (EMSA) to a β-casein promoter sequence. The STAT5 DNA-binding activity of nuclear protein was increased at both 5 and 60 min after GH treatment of CWSV-1 cells (Fig. 6). Pretreatment of cells with TNF-α did not affect basal STAT5 DNA-binding activity of nuclear protein but diminished the GH-induced STAT5 DNA-binding activity at 60 min. The specificity of the STAT5 binding was confirmed by addition of cold consensus and mutated competitor oligonucleotides and by the ability of antibody specific for STAT5b to shift the mobility of the STAT5-DNA complex as shown in Fig. 6, lanes 7–9.

**DISCUSSION**

GH is an important mediator of growth, differentiation, and metabolism in many tissues (30). The somatomedin hypothesis proposes that circulating IGF-I is synthesized by the liver in response to GH and is responsible for the effects of GH on postnatal growth and development. Consequently, GH administration has been suggested as an adjunct therapy to nutritional support to prevent the erosion of lean body mass observed in patients with catabolic diseases such as sepsis and burn injury (52). However, in 1988, Dahn et al. (13) demonstrated that neither circulating IGF-I nor urinary nitrogen excretion was improved in septic patients treated with rhGH. The results of that and other studies (12, 21, 29, 34) suggested that systemic infection and other catabolic illnesses are associated with the development of a GH-resistant state.

The inflammatory cytokines (TNF-α, IL-1β, and IL-6) have been proposed as potential mediators of GH resistance. Intravenous injection of TNF-α and IL-1β in healthy animals results in a 30–40% reduction in circulating and tissue IGF-I levels (18, 20). Furthermore, treatment of septic rats with IL-1 receptor antagonist (IL-1ra) attenuates the effects of sepsis on the GH/IGF-I system (34). Treatment of septic rats with TNFbp attenuates the catabolism of muscle protein and ameliorates the sepsis-induced inhibition of gastrocnemius protein synthesis (11). In the present...
study, injection of TNFbp ameliorated the increase in circulating GH and reduction in plasma IGF-I observed in septic rats. Although septic animals demonstrate reduced food intake on days 1–3, chow consumption returned to normal on days 4–5 and was not influenced by TNFbp administration (9, 11). Furthermore, plasma levels of corticosterone are similar in septic and control rats on day 5 after chronic abdominal sepsis (19, 36). Consequently, neither differences in circulating glucocorticoid levels nor those in food intake appear to explain the effects of TNFbp on the GH/IGF-I axis in septic animals.

In the present study, TNFbp was administered before the induction of sepsis to ensure that inhibitory levels of the TNF antagonist would coincide with peak plasma TNF levels (9, 11). Although circulating levels of TNF receptor are present after endotoxemia in humans (53), they are preceded by a peak in plasma TNF. Because only a brief exposure to TNF appears to be required to induce GH resistance in hepatocytes, the timing of TNFbp administration may be an important determinant in its ability to ameliorate the hepatic GH resistance.

The decrease in circulating IGF-I observed in septic rats was accompanied by reductions in hepatic IGF-I and GHR mRNA. The ability of TNFbp to ameliorate the inhibitory effects of sepsis on IGF-I and GHR mRNA levels in liver suggests that TNF is an important in vivo mediator of this phenomenon. In contrast to liver, the abundance of IGF-I mRNA in gastrocnemius was not significantly altered by sepsis or TNFbp. This may reflect a relative resistance of muscle to the inhibitory effects of sepsis on IGF-I synthesis compared with liver. Previous studies from our laboratory demonstrate a 30% reduction in IGF-I protein levels in gastrocnemius from septic rats relative to controls. A concomitant 40% reduction in protein synthesis was also observed in gastrocnemius from septic rats. This observation may help to explain the discrepancy between IGF-I mRNA and protein levels observed in gastrocnemius during chronic abdominal sepsis.

The results of several studies suggest that the effects of sepsis and inflammatory cytokines on the GH/IGF-I axis are mediated by decreased expression or affinity of the GHR for its ligand (15, 16, 50). In primary rat hepatocytes, both TNF-α and IL-1β inhibited the synthesis of IGF-I mRNA in response to GH (50). Defalque et al. (15) found that, in endotoxin [lipopolysaccharide (LPS)]-treated rats, hepatic GHR mRNA and GHR-binding activity were reduced 5 h after the insult, suggesting that the LPS-induced GH resistance is caused by a decreased expression of hepatic GHR. A reduction in hepatic GHR mRNA was also observed in LPS-treated wild-type but not LPS-treated TNF receptor knockout mice (16). In this study, TNF was implicated in the regulation of GHR expression at the transcriptional level by inhibiting Sp1 and Sp3 transactivation of the GHR promoter (16).

To determine whether decreased expression of GHR may be responsible for the reduction in circulating IGF-I observed in septic rats, the relative abundance of GHR protein in hepatic membrane preparations from Control, Sepsis, and Sepsis + TNFbp animals was measured. Neither sepsis nor TNFbp significantly altered the relative amount of GHR protein. Consequently, the GH-resistant state observed with abdominal sepsis does not appear to be caused by a reduction in the amount of hepatic GHR. These results are similar to those observed by Mao et al. (38) in LPS-treated rats in which similar levels of GHR protein were noted in liver from control and LPS-treated rats 4 h after LPS administration. Although differences in the abundance
of hepatic IGF-I mRNA were reflected in the circulating IGF-I protein levels, the same relationship between mRNA and protein was not observed for the GHR mRNA and protein. Although the reduction in hepatic GHR mRNA could result in decreased synthesis of GHR protein, the relative abundance of GHR would remain unchanged if GHR stability or degradation were influenced as well. Recent observations suggest that GHR expression may be controlled, at least in part, by translational mechanisms (28). This might explain the discrepancy observed between the mRNA and protein levels in liver under in vivo conditions.

To investigate whether the effects of TNF-α on hepatic GH resistance were directly attributable to TNF-α, we performed additional experiments in a cultured rat hepatocyte model. The CWSV-1 hepatocytes were chosen because the cells express GHR, synthesize IGF-I in response to GH (32, 37, 54), and transduce GH signals via the JAK/STAT pathway (22, 32, 45). Incubation of hepatocytes with TNF-α for 4 h completely inhibited the synthesis of IGF-I mRNA induced by GH but had no effects on IGF-I mRNA content under basal, nonstimulated conditions. Treatment of CWSV-1 cells with TNF-α had no effect on the relative abundance of GHR protein. This finding confirms our in vivo results in septic rats and provides additional evidence that decreased expression of GHR is not necessary for the induction of GH resistance by TNF-α. However, there is a significant reduction in the levels of tyrosine-phosphorylated GHR observed 5 min after GH administration in TNF-treated cells. These data suggest that TNF-α attenuates the duration of the JAK/STAT signaling by GH.

The activated GHR complex stimulates multiple signaling pathways including Ras-mitogen-activated protein kinase, insulin receptor substrate-phosphatidylinositol 3-kinase, and JAK/STAT (26). Although the mechanism(s) by which GH stimulates IGF-I synthesis is unknown, mice with targeted deletion of STAT5 demonstrate reduced basal and GH-induced levels of plasma IGF-I (14). Previous studies suggest that LPS administration inhibits hepatic GH signaling via the JAK2/STAT5 pathway (3, 38). We therefore examined the effects of TNF-α on STAT5 tyrosine phosphorylation, nuclear translocation, and the STAT5-dependent β-casein promoter DNA binding.

The comparable degree of STAT5 activation (STAT5 tyrosine phosphorylation, nuclear translocation, and DNA binding) observed 5 min after GH administration in TNF-α and control cells shows that TNF-α does not completely prevent GH signaling via the JAK/STAT pathway in CWSV-1 hepatocytes. However, the reduction in STAT5 activation observed 60 min after GH administration in TNF-treated cells suggests that TNF-α significantly attenuates the duration of JAK/STAT5 signaling by GH in these cells. The relative abundance of STAT5 was similar in cytoplasmic cell lysates from TNF-α and control cells at both 5 and 60 min after GH. Consequently, reductions in total STAT5 do not explain the decrease in STAT5 activity observed at 60 min after GH administration in the TNF-treated cells. Instead, the time course of STAT5 activation-deactivation that was noted suggests that TNF-α pretreatment enhances the termination of GH-induced STAT5 signaling.

Several biochemical events appear to be important in regulating the duration of GH-induced STAT5 signaling. The activated GHR complex is internalized by endocytosis and subsequently degraded by the ubiquitin-proteasome system (2). Administration of the proteasome inhibitor MG132 has been shown to prolong the GH-induced activity of GHR, JAK2, and STAT5 by stabilizing the tyrosine phosphorylation status of these proteins (2, 23). TNF-α has been shown to activate ubiquitin-dependent proteasome-mediated proteolytic pathways (35, 41). Thus the inhibitory effects of TNF-α on GH signaling could be caused by proteasome-mediated deactivation of the GH signal.

The duration of GH-activated STAT5 signaling also depends on the activity of JAK2 (which phosphorylates STAT5) as well as the dephosphorylation of STAT5 by phosphotyrosine phosphatases (reviewed in Refs. 26 and 27). The phosphorylation status and kinase activity of JAK2 were not measured in the present study; however, both the GHR and STAT5 are tyrosine phosphorylated by JAK2 (27). Therefore, the reductions in GH phosphorylation (5 min) and STAT phosphorylation (60 min) observed in TNF-treated cells could be explained by reductions in activity of JAK2, because the relative abundance of JAK2 was unaltered (data not shown). An alternative explanation is that an increase in phosphotyrosine phosphatase activity is responsible for the inhibitory effects of TNF-α on GH signaling. Phosphotyrosine phosphatase inhibitors block the deactivation of STAT5b after termination of a GH pulse (23). Dephosphorylation of GHR, JAK2, and STAT proteins is thought to involve the recruitment of one or more SH2 tyrosine phosphatases to GHR/JAK2 or JAK/STAT complexes. GH stimulation induces SHP-1 phosphatase activity, activates nuclear translocation, and promotes its association with phosphorylated nuclear STAT5b in CWSV-1 cells (23). Consequently, the activity of SHP-1 and other tyrosine phosphatases represents another important regulatory mechanism for STAT5b signaling which could be influenced by TNF-α.

The recently described suppressors of cytokine signaling (SOCS) have also been shown to inhibit GH-mediated signaling through interactions with the cytoplasmic portion of the GHR and/or JAK2 (8, 25, 33). The messenger RNA for cytokine-inducible Sh2 protein (CIS) or SOCS1–3 is typically found at low levels in different cells or tissues (4, 8, 33). However, an increase in SOCS mRNA is commonly observed when cells are exposed to various cytokines or growth factors, including TNF-α and GH (8). Overexpression of the SOCS proteins in transfected cells inhibits the activation of Spi 2.1 and the acid-labile subunit by GH (3, 4, 25). Furthermore, LPS-treated rats demonstrate a fourfold induction of CIS mRNA and a tenfold induction of SOCS-3 mRNA in liver when compared with saline-treated controls (38). A recent study showed
that TNF-α could potentiate the induction of SOCS-3 and CIS by GH in primary rat hepatocytes (4). Thus TNF-mediated induction of SOCS represents another potentially important mechanism by which sepsis or inflammatory cytokines could inhibit GH signaling.

In summary, TNF-α appears to be an important mediator of the GH-resistant septic state. Reductions in hepatic GHR are not necessary to explain the inhibitory effects of sepsis or TNF-α on the induction of IGF-I synthesis by GH. Although TNF-α does not appear to impair early GH signaling, this cytokine clearly inhibits the duration of GH-mediated JAK/STAT signaling. Several regulatory mechanisms could explain the inhibition of hepatic GH signaling observed in TNF-treated cells: proteasome-mediated deactivation of the GHR/JAK2 complex, stimulation of tyrosine phosphatase activity, or the inhibition of JAK/STAT signaling by SOCS. Additional studies will be necessary to determine the relative importance of these different biochemical pathways in regulating hepatic JAK/STAT signaling by TNF-α and GH.

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REFERENCES


