Endotoxin-Induced Inhibition of Growth Hormone Receptor Signaling in Rat Liver in Vivo*

YILEI MAO, PEI-RA LING, TIMOTHY P. FITZGIBBONS, KAREN C. MCCOWEN, G. PETER FRICK, BRUCE R. BISTRIAN, AND ROBERT J. SMITH

Joslin Diabetes Center (Y.M., T.P.F., K.C.M., R.J.S.) and Beth Israel-Deaconess Medical Center (P.-R.L., K.C.M., B.R.B., R.J.S.), Harvard Medical School, Boston, Massachusetts 02215; and the Department of Physiology, University of Massachusetts Medical School (G.P.F.), Worcester, Massachusetts 01655

ABSTRACT

The bacterial lipopolysaccharide endotoxin induces a catabolic response characterized by resistance to multiple anabolic hormones. The objective of this study was to determine the effects of endotoxin on the GH signaling pathway in rat liver in vivo. After the iv injection of Escherichia coli endotoxin (1 mg/kg), there was a progressive decrease in liver STAT5 (signal transducer and activator of transcription-5) tyrosine phosphorylation in response to GH (40% decrease 6 h after endotoxin), which occurred in the absence of a change in abundance of the STAT5 protein. Endotoxin resulted in a rapid 40-fold increase in liver Janus family kinase-2 (JAK2) messenger RNA, followed by a 2-fold increase in JAK2 protein abundance. This was associated with a 50% decrease in phosphorylated/total JAK2 after endotoxin infusion. The finding of endotoxin inhibition of in vivo STAT5 tyrosine phosphorylation in response to a supramaximal dose of GH in the absence of a change in GH receptor abundance or total GH-stimulated JAK2 tyrosine phosphorylation provides the first demonstration of acquired postreceptor GH resistance. We hypothesize that this may occur through a specificity-spillover mechanism involving the induction of SOCS genes by cytokines released in response to endotoxin and subsequent SOCS inhibition of GH signaling. (Endocrinology 140: 5505–5515, 1999)

RESISTANCE TO GH has been demonstrated in the liver and other tissues after bacterial sepsis, trauma, burns, major surgery, hypoglycemia, or starvation (1–3). Consistent with hepatic resistance to endogenous GH, insulin-like growth factor-I (IGF-I) production and plasma IGF-I concentrations, which are determined largely by GH-induced IGF-I synthesis in the liver (2, 4, 5), are decreased in critically ill and septic patients despite elevated plasma GH levels (6, 7). This GH resistance may contribute to the catabolic response that is characteristic of severe illness and, ultimately, to an unfavorable disease outcome. The administration of GH to critically ill patients has been shown to promote tissue anabolism under some circumstances, but exogenous GH may produce untoward side-effects and is not uniformly capable of inducing an anabolic response (8, 9). Therefore, it is important to understand the mechanism of GH resistance in catabolic states.

The immediate signaling events following GH binding to its receptor have only recently been defined (10). In target tissues, such as liver and skeletal muscle, GH binding results in dimerization of the GH receptor and consequent activation of the intracellular receptor-associated tyrosine kinase Janus kinase 2 (JAK2) (11, 12). This is followed by JAK2 autophosphorylation and tyrosine phosphorylation of the GH receptor and members of the signal transducer and activator of transcription (STAT) protein family (13). Phosphorylation of the STAT proteins leads to their dimerization and translocation to the nucleus, where they modulate gene transcription (10, 14). Although studies in cell culture and experimental animals have provided evidence for GH-stimulated tyrosine phosphorylation of a number of cellular signaling proteins, including various STATs (10, 15), insulin receptor substrate-1 (IRS-1), IRS-2, IRS-3 (13, 16, 17), and the SHC proteins (18), STAT5 appears to be the dominant intracellular signaling protein phosphorylated by GH-activated JAK2 in adult rat liver and muscle in vivo (12, 14). GH resistance in catabolic states has been related at least in part to decreased tissue abundance of GH receptors (19–21). In other hormonal pathways, such as the insulin signaling pathway, disease-related hormone resistance has been shown to result from changes in both receptor abundance and postreceptor signaling responses (22–24). It is not known whether GH resistance also may result from alterations in postreceptor steps in the GH signaling pathway.

In many experimental studies, infusion of the Gram-negative bacterial cell wall lipopolysaccharide, endotoxin, has been used to generate a model of catabolic disease states. Endotoxin is an important contributor to the fever and tissue

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Address all correspondence and requests for reprints to: Robert J. Smith, M.D., Joslin Diabetes Center, Harvard Medical School, One Joslin Place, Boston, Massachusetts 02215. E-mail: robert.smith@joslin.harvard.edu.

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catabolic response associated with Gram-negative sepsis (2, 3). In normal humans injected with *Escherichia coli* endotoxin, plasma IGF-I concentrations were shown to decrease despite a transient, marked increase in GH levels (25), suggesting the development of GH resistance. Studies in experimental animals have demonstrated that infusion of endotoxin results in marked decreases not only in plasma IGF-I concentrations, but also in IGF-I messenger RNA (mRNA) abundance in plasma, liver, and skeletal muscle (26). A decrease in circulating IGF-I levels after endotoxin infusion also was evident in rats treated with exogenous GH (27), further supporting the hypothesis that endotoxin induces a state of GH resistance. Endotoxin has been shown to induce postreceptor insulin resistance characterized by a marked decrease in insulin-stimulated tyrosine phosphorylation of the insulin receptor and the intracellular receptor substrate IRS-1 (24). The possibility that endotoxin leads to abnormalities in the content or tyrosine phosphorylation of JAK2 and intermediates in the GH signaling pathway has not been investigated.

As potential mediators of endotoxin-induced GH resistance, a number of related genes, designated suppressors of cytokine signaling (SOCS), that are believed to have a role in JAK/STAT signal attenuation recently have been identified (28–30). Interleukin-6, leukemia inhibitory factor, erythropoietin, and leptin, in addition to GH, have been shown to rapidly increase expression of SOCS genes in *vivo* and *in vitro* through STAT-mediated transcriptional activation (29, 31–36), and the protein products of the SOCS genes are thought to serve as negative feedback inhibitors by decreasing JAK/STAT signaling. As the expression of SOCS genes can be stimulated by multiple cytokines, we considered the possibility that increased SOCS gene expression induced by one or more cytokines secreted as part of the response to endotoxin infusion could result in resistance to GH.

In this study we have determined the effects of endotoxin infusion on the tyrosine phosphorylation and tissue content of proteins mediating early steps in the GH signaling pathway in rat liver *in vivo*, including JAK2, STAT5, and the GH receptor. To investigate SOCS gene expression as a potential molecular mechanism of endotoxin-induced GH resistance, rat complementary DNAs (cDNAs) corresponding to the full coding sequences of three members of this gene family [cytokine-inducible sequence (CIS), SOCS-2, and SOCS-3] were cloned and used as probes to define the levels of their corresponding mRNAs in liver tissue.

**Materials and Methods**

E. coli endotoxin (BE colo 026:B6 lipopolysaccharide) was obtained from Difco (Detroit, MI). Recombinant human GH (hGH) was a gift from Genentech, Inc. (South San Francisco, CA). JAK2 polyclonal antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). STAT5 monoclonal antibody was obtained from Transduction Laboratories, Inc. (Lexington, KY). Polyclonal phosphotyrosine antibodies (37) and anti-GH receptor antibodies (38) were prepared as described previously.

**Endotoxin and GH infusion protocols**

Male Sprague Dawley rats (Taconic Farms, Inc., Germantown, NY), weighing 180–200 g, were acclimated for 5 days in a light-controlled room at 24 C, with water and chow available ad libitum. Nonfasted animals were anesthetized with ketamine (40 mg/kg BW, ip), and under pentobarbital anesthesia a Silastic (Dow Corning Corp., Midland, MI; id, 0.025 in.; od, 0.047 in.) was implanted in the right jugular vein through a small incision, tunneled sc to the dorsal interscapular region, and exteriorized by suturing to a rotating swivel device (Instech Laboratories, Plymouth Meeting, PA). After recovery from anesthesia, the animals were placed in individual metabolic cages with the catheter assembly anchored in a manner enabling free movement around the cage. Water and chow were provided ad libitum, and the catheters were flushed every 2 days with a small volume of heparinized saline to maintain patency.

Four days after catheter implantation, nonfasted rats were given a bolus iv injection of either endotoxin (1 mg/kg BW) or saline through the jugular venous catheter. This represents a dose of endotoxin that is known to induce insulin resistance but to be sublethal during the planned period of study (24). At various time points after endotoxin or saline administration (0–6 h), the animals were given a bolus injection of either hGH (1.5 mg/kg BW) or saline through the jugular catheter. Previous studies have shown marked stimulation of GH signaling responses in rats after the infusion of recombinant human GH (12, 39). The dose of 1.5 mg/kg used in this study was selected because it is known to be approximately 10-fold greater than the amount required to produce a maximum response, and it is known to result in the same effects on the activation of GH signaling intermediates as lower doses. As described in previous reports on GH signaling (12, 39) and insulin signaling in *vivo* (24, 37, 40), supramaximal doses of hormones are used in this type of experimental system to assure that observed changes in signaling do not result from altered hormone delivery to tissue receptors. Five minutes after GH or saline injection, the animals were decapitated, and the left lobe of the liver was rapidly removed, frozen in liquid nitrogen, and stored at −80 C for subsequent analysis. It is known from previous *in vivo* studies that 5 min of GH stimulation results in maximum tyrosine phosphorylation of major GH signaling proteins (12). The animal protocols were in compliance with the Guide for the Care and Use of Laboratory Animals published by the NIH and were approved by the institutional animal care committee.

**Immunoprecipitation and immunoblotting**

Frozen liver tissue samples were weighed, pulverized under liquid nitrogen with a stainless steel mortar and pestle, and homogenized with a Polytron (Brinkmann Instruments, Inc., Westbury, NY) in 6 vol buffer containing 20 mM Tris-HCl (pH 7.6), 120 mM NaCl, 1% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethanesulfonylfluoride, 40 μg/ml leupeptin, and 100 mM sodium fluoride. The resulting homogenates were centrifuged at 200,000 × g for 1 h at 4 C, and the supernatants were collected and stored in aliquots at −80 C. The protein concentrations of the tissue extracts were determined with a Bradford dye binding assay kit (Bio-Rad Laboratories, Inc., Hercules, CA), using BSA as a standard.

Equal amounts of tissue protein (5 mg) were incubated in the above-described buffer with specific antibodies at the indicated dilutions overnight at 4 C. The antibodies were then adsorbed onto protein A-Sepharose beads (Pierce Chemical Co., Rockford, IL) for 2 h at 4 C, and the resulting immunocomplexes were washed three times by centrifugation and resuspension in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (vol/vol) Nonidet P-40, 1 mM EDTA, 0.25% (wt/vol) sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM sodium fluoride. The washed immunoprecipitates plus additional tissue extracts (200 μg) not subjected to immunoprecipitation were heated in Laemmli buffer with 100 mM dithiothreitol for 5 min at 100 C, resolved by SDS-PAGE, and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). The membranes were blocked with 20 mM Tris buffer (pH 7.4) containing 0.9 g/dl sodium chloride, 15 mM sodium azide, 5% (wt/vol) BSA, and 0.05% (vol/vol) Nonidet P-40 and probed with the indicated antibodies. After washing, specifically bound antibodies were detected with [125I]protein A as previously described (37) and quantitated with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).
Cloning of rat SOCS cDNAs

As rat cDNAs for members of the SOCS gene family were not available, and the rat sequences for these genes had not been published, we cloned cDNAs containing the full coding sequences of three SOCS genes, CIS, SOCS-2, and SOCS-3, by RT of total RNA from rat liver, followed by PCR amplification with primers corresponding to the equivalent mouse cDNA sequences. For this purpose, 5-μg aliquots of total RNA were incubated in 20-μl vol at 42°C with random primers (Life Technologies, Inc., Gaithersburg, MD) and 100 U Moloney murine leukemia virus H reverse transcriptase (Superscript, Life Technologies, Inc.). Using 100-ng cDNA equivalents of the RT products as template, 30 cycles of PCR were conducted with 18- to 20-mer primers (Joslin DNA Core Facility, Boston, MA) at 0.15 mM with 2.5 U Pfu DNA polymerase (Stratagene, La Jolla, CA) and the following parameters: 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The resulting cDNAs were restriction digested and cloned into the pGEX vector (Amersham Pharmacia Biotech, Piscataway, NJ). Multiple clones corresponding to each SOCS family mRNA were sequenced several times in both directions to assure correct definition of the rat sequence.

Northern blotting

Total liver RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (41). Final RNA concentrations were determined spectrophotometrically at 260 nm, and the samples were stored in aliquots at −80°C. For Northern blotting, 10- to 30-μg aliquots of denatured RNA from each sample were resolved by electrophoresis in 1.2% agarose. RNA integrity and equality of loading were confirmed by analysis of ethidium bromide-stained 28S and 18S ribosomal RNA bands. The RNA then was transferred to GeneScreen membranes (Life Science Products, Boston, MA) by overnight blotting in sodium chloride-sodium citrate (SSC) and fixed with a UV cross-linker (Stratagene, La Jolla, CA). The membranes were prehybridized for 2–4 h at 42°C in 50% (vol/vol) formamide, 10% (wt/vol) dextran sulfate, and 1% (wt/vol) SDS.

Hybridization was performed for 14–16 h at 42°C in prehybridization solution supplemented with 0.1 mg/ml denatured salmon sperm DNA using specific rat cDNA probes labeled with [32P]dCTP (~2×10^6 dpm/ml) by random priming (Multiprime DNA Labeling Kit, Amersham Pharmacia Biotech, Arlington Heights, IL). These included an approximately 2000-bp JAK2 cDNA (provided by Dr. Roy Duhe, NCI-Frederick Cancer Research and Development Center, Frederick, MD) and an 807-bp CIS cDNA, a 920-bp SOCS-2 cDNA, and an 831-bp SOCS-3 cDNA cloned in our laboratory. After hybridization, the membranes were sequentially washed for 5 min with 2×SSC-0.1% SDS and 1×SSC-0.1% SDS at 22°C and then with 0.1×SSC-0.1% SDS at 42°C. Specific mRNA bands were identified and quantified using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Each blot was subsequently stripped and rebotted with a mouse 18S ribosomal cDNA probe to verify equal total RNA loading. To determine the time course of the effects of endotoxin on the different mRNAs, only samples from rats treated for 5 min with GH were analyzed. It can be reliably assumed that mRNA levels would not be affected by the short period of GH treatment, and this limited the total number of assays required.
**Results**

**STAT5 tyrosine phosphorylation and tissue content**

Previous studies have demonstrated a rapid marked increase in STAT5 tyrosine phosphorylation in rat liver and other tissues in vivo in response to GH (12), and this signaling response is believed to have an important role in mediating the regulatory effects of GH on gene expression. To determine whether endotoxin administration results in decreased GH signaling, we initially studied rats 4 h after a bolus iv dose of E. coli endotoxin (1 mg/kg BW) or saline. In control nonendotoxin-treated rats, 5 min of GH treatment resulted in a marked increase in the tyrosine phosphorylation of a single, dominant, 95-kDa protein band in liver extracts (Fig. 1A, left panel), which previously has been shown to represent STAT5 (12). Quantitative analysis of immunoblots from multiple control rats demonstrated a 7-fold increase in STAT5 tyrosine phosphorylation after GH stimulation (Fig. 1A, right panel). Treatment with endotoxin had no effect on the basal level of phosphorylation of STAT5 in the absence of GH, but resulted in a significantly decreased stimulatory effect of GH on STAT5 tyrosine phosphorylation (60% of GH-stimulated control value; \( P < 0.05 \)).

To determine whether endotoxin treatment altered the tissue content of STAT5, liver extracts from the same animals were analyzed by immunoblotting with STAT5 antibody. As shown in Fig. 1B (left panel), STAT5 antibody bound to two protein bands of approximately 95 and 92 kDa. Previous studies from our group and others have demonstrated that the upper band represents tyrosine-phosphorylated STAT5, and the lower band represents nonphosphorylated STAT5 (12, 17). In accordance with this conclusion, GH stimulation resulted in an increase in the higher molecular mass band and a corresponding decrease in the lower band in liver from both control and endotoxin-treated animals. When the total content of STAT5 was determined by quantifying the densities of the two bands on immunoblots from multiple rats, no differences were observed in control vs. endotoxin-treated rats in the basal state or in control vs. endotoxin-treated rats after GH stimulation (Fig. 1B, right panel). There was a small, but significant, decrease in the intensity of the combined STAT5 bands in GH-stimulated vs. basal rats in both the presence and absence of endotoxin treatment, which was attributed to a presumed decrease in the affinity of the STAT antibody for phosphorylated compared with nonphosphorylated STAT5. When considered together with the results presented in Fig. 1A, these data indicate that endotoxin administration leads to a decrease in GH-stimulated liver STAT5 tyrosine phosphorylation that is not explained by altered tissue content of the STAT5 protein.

**Endotoxin-induced GH resistance**

To evaluate the time course of the inhibitory effects of endotoxin on STAT5 tyrosine phosphorylation, rats were injected iv with hGH (1.5 mg/kg) at various time intervals (0.5–6 h) after a bolus iv injection of endotoxin (1 mg/kg), and liver tissue was removed for analysis 5 min after GH administration. To provide measures of the effects of GH in the absence of endotoxin, STAT5 phosphotyrosine levels
were determined in basal and GH-stimulated rats not treated with endotoxin and in additional rats given a bolus injection of saline and studied in the basal and GH-stimulated states 6 h later. As shown in a representative immunoblot (Fig. 2, left panel) and by quantitative results from multiple animals (Fig. 2, right panel), there was a progressive time-dependent decrease in GH-stimulated STAT5 tyrosine phosphorylation throughout the 6-h study period after endotoxin administration. Although saline controls were not included at each time point, it is reasonable to conclude that the decrease in STAT5 phosphorylation resulted from the effects of endotoxin, because the injections were given to unanesthetized rats via implanted catheters with little disturbance of the animals. In support of this conclusion, there was no change in basal or GH-stimulated STAT5 phosphorylation 6 h after administration of a saline control solution, and a similar lack of change in control animals was demonstrated 4 h after endotoxin in the study described in Fig. 1.

**JAK2 tyrosine phosphorylation and tissue content**

The effect of endotoxin on JAK2 tyrosine phosphorylation was determined as a measure of a GH signaling response proximal to STAT5 phosphorylation. For this purpose, the same liver extracts used for the previously described STAT5 studies were analyzed by sequential immunoprecipitation with JAK2 antibody followed by immunoblotting with phosphotyrosine antibody. As with STAT5 phosphorylation, there was a marked increase in tyrosine phosphorylation of the 130-kDa JAK2 protein band 5 min after GH administration (Fig. 3A). During the first 1–2 h after the infusion of endotoxin, there was a trend toward decreased GH-stimulated tyrosine phosphorylation of JAK2, but this did not reach statistical significance at any time point compared with the GH-stimulated 0 and 6 h saline control values. There was no change in basal or GH-stimulated JAK2 phosphorylation in the 6 vs. 0 h saline controls (Fig. 3A) and no change in 4-h saline-infused animals in a separate study (data not shown).

To determine whether endotoxin affects the tissue abundance of JAK2, the content of the JAK2 protein was determined by sequential immunoprecipitation and immunoblotting with JAK2 antibody using the same liver extracts from rats treated for different periods with endotoxin (Fig. 3B). There was no change in JAK2 during the first 2 h after endotoxin; at the 4 h point, there was a 2-fold increase in JAK2 protein content, which was sustained 6 h after endotoxin (P < 0.01). In control rats infused with saline instead of endotoxin, JAK2 content was unchanged after 6 h (Fig. 3B), and a similar lack of change in JAK2 content was demonstrated 4 h after endotoxin in a separate group of animals (data not shown). When the ratio of tyrosine-phosphorylated
JAK2 to total JAK2 was calculated in individual GH-stimulated rats, a progressive, time-dependent decrease in endotoxin-treated animals was evident, such that the relative stoichiometry of JAK2 phosphorylation was 31% of the level in controls 6 h after endotoxin administration (P < 0.05; Fig. 4).

**JAK2 mRNA levels**

To investigate the basis for the endotoxin-induced increase in tissue content of the JAK2 protein, JAK2 mRNA levels were determined in liver total RNA preparations by Northern blotting. As previously reported (42), a predominant JAK2 mRNA transcript of approximately 5 kb and a less abundant, slightly smaller transcript were identified. Endotoxin infusion resulted in a marked increase in the levels of both of these mRNAs (Fig. 5, left panel). Quantitative analysis of the combined JAK2 transcripts on Northern blots from multiple animals demonstrated that the increase in JAK2 mRNA was detectable at 1 h, reached a maximum (40-fold above control) at 2 h, and was sustained for at least 6 h after a single dose of endotoxin. There was no change in JAK2 mRNA in saline-injected control animals studied at the 0 vs. 6 h points. From these data, it can be concluded that the increase in JAK2 protein in the liver after endotoxin is preceded by an even more marked increase in JAK2 mRNA.

**GH receptor abundance**

Because changes in GH responsiveness could be explained by altered tissue content of the GH receptor, the amount of receptor protein in the livers of control and endotoxin-treated rats was determined. For this purpose, the same tissue extracts used for the studies on GH signaling were analyzed by sequential immunoprecipitation and immunoblotting with GH receptor antibody. As shown in Fig. 6 (left panel), the GH receptor was identified as a broad band of approximately 110 kDa. Quantitation of immunoblots from multiple animals demonstrated no differences in liver GH receptor content between control and endotoxin-treated rats (Fig. 6, right panel). Thus, the decreased effects of GH on STAT5 tyrosine phosphorylation and on the stoichiometry of JAK2 phosphorylation after endotoxin treatment cannot be attributed to a decreased abundance of GH receptors. It was not possible to determine the effects of GH stimulation or endotoxin pretreatment on GH receptor tyrosine phosphorylation, because of an apparent decreased effectiveness of the GH receptor antibody in precipitating receptors from rats after GH administration (data not shown).

**SOCS gene expression**

As a potential mechanism to explain the inhibitory effects of endotoxin on GH signaling responses, the steady state levels of mRNAs corresponding to three members of the SOCS gene family (CIS, SOCS-2, and SOCS-3) were determined in liver total RNA extracts. Multiple SOCS genes recently have been identified as negative feedback inhibitors of cytokine receptor signaling (28, 43), and CIS, SOCS-2, and SOCS-3 were selected because they are relatively abundant in liver. Rat cDNAs containing the full coding regions of these genes were cloned [see Materials and Methods and GenBank entries (see Footnote 1) for further details]. In brief, the rat DNA sequences are approximately 95% identical to the corresponding mouse sequences and 90% identical to human sequences. At the amino acid level, the homologies are approximately 97% and 94% for mouse and human, respectively.

As shown in Fig. 7A, a single CIS mRNA transcript of 2.5 kb was identified in rat liver. This mRNA was almost undetectable in liver from control animals and increased markedly after endotoxin administration. A significant increase in CIS mRNA was evident 1 h after endotoxin infusion; this increase reached a maximum of 4-fold above the control value 2 h after endotoxin. The SOCS-2 cDNA probe hybridized with a 3.4-kb mRNA transcript, which also was present at low levels in liver from control rats and increased after endotoxin (Fig. 7B). The more slowly migrating band evident in Fig. 7B was not observed in additional experiments and thus is not thought to represent a SOCS-2 mRNA transcript. Quantitation of the 3.4-kb transcript in multiple animals demonstrated a small, transient increase in SOCS-2 mRNA, which was significant only at the 1 h point after endotoxin administration (Fig. 7B, right panel). SOCS-3 mRNA was most markedly affected by endotoxin treatment. A single 3.2-kb SOCS-3 transcript identified with the rat cDNA probe was present at a low level in control rat liver, increased approximately 8-fold at 1 h and 10-fold at 2 h after endotoxin treatment, and remained significantly elevated for at least 6 h after a single dose of endotoxin (6.4-fold at 6 h). There were no differences in mRNA levels for these three SOCS members

![Fig. 4](https://academic.oup.com/endo/article-abstract/140/12/5505/2990403/Downloaded_from_73/42605286493)
in saline-injected control animals studied at 0 and 6 h. Thus, coincident with the induction of GH resistance by endotoxin, there was a marked increase in hepatic mRNA levels for CIS, SOCS-2, and SOCS-3. These and other members of the SOCS gene family represent potential mediators of the endotoxin-induced alterations in GH-stimulated STAT5 and JAK2 tyrosine phosphorylation described in this study.

**Discussion**

Published studies in humans and experimental animals have provided evidence for the development of GH resistance in severe illness (1, 2, 25). In this report, using an experimental model of *E. coli* endotoxin infusion in laboratory rats, we have defined specific endotoxin-induced abnormalities in the activation of signaling intermediates in the liver after GH administration in vivo. After the iv injection of a sublethal dose of endotoxin, the capacity of a maximally effective dose of GH to stimulate STAT5 tyrosine phosphorylation decreased rapidly. Analysis of the time course of the endotoxin effect demonstrated a progressive decline in GH-stimulated STAT5 phosphorylation for at least 6 h after a single endotoxin injection, with a resulting 60% decrease in the amount of tyrosine-phosphorylated STAT5 at the 6-h point. Under the same conditions, there was no change in the amount of STAT5 protein in liver extracts, indicating that endotoxin decreased the extent of STAT5 phosphorylation rather than the amount of STAT5 available for phosphorylation. As STAT5 phosphorylation correlates with its activity in regulating gene transcription, and STAT5 is a key mediator of the actions of GH (12, 17), the observed decrease in GH-stimulated STAT5 phosphorylation provides a potential molecular mechanism to explain GH resistance induced by endotoxin or other catabolic states.

The binding of GH to its cell surface receptors activates the receptor-associated JAK2 tyrosine kinase (10), which then undergoes autophosphorylation and catalyzes the phos-
phorylation of tyrosine residues in a number of cellular proteins, including the GH receptor and STAT5 (12, 13). At various time points after endotoxin administration, there was a 15–50% decrease in mean JAK2 tyrosine phosphorylation after GH stimulation, but this did not reach statistical significance because of relatively large interanimal variation in both control and endotoxin-treated rats. By contrast, endotoxin resulted in a highly significant, greater than 2-fold increase in the liver content of JAK2, which became apparent 4 h after endotoxin and persisted through the 6 h point. When the ratio of JAK2 tyrosine phosphorylation to JAK2 protein content was determined in individual animals, a progressive, time-dependent decrease in the mean stoichiometry of JAK2 phosphorylation induced by endotoxin was evident (69% decrease 6 h after endotoxin). Autophosphorylation occurs on multiple tyrosine residues in JAK2 (44), and thus, the decrease in JAK2 phosphorylation could result from decreased phosphorylation of individual sites within JAK2 or...
an increase in the pool of nonphosphorylated JAK2. Further investigation of these two alternative mechanisms and their implications for JAK2 signaling to STAT5 will probably require the development of an *in vitro* experimental system in which radioisotopic tracer methodology can be used to map the effects of endotoxin on specific JAK2 tyrosine phosphorylation sites.

The increase in JAK2 protein content induced by endotoxin was preceded by a marked rise in the level of JAK2 mRNA transcripts in the liver. JAK2 mRNA was elevated within 1 h of endotoxin administration, increased 40-fold 2 h after endotoxin, and remained elevated for at least the next 4 h. Although the half-life of JAK2 mRNA in the liver or other mammalian tissues has not been established, it is unlikely that the rapid change in JAK2 mRNA can be explained by mRNA stabilization, and it is probable that this represents an endotoxin-induced increase in JAK2 gene transcription. JAK2 mRNA has been reported to be rapidly up-regulated after mitogenic stimulation of B lymphocytes and, to a lesser extent, T cells (45). However, to our knowledge, the response to endotoxin described in the present paper represents the first example of rapid JAK2 regulation observed in an *in vivo* and in a nonlymphocyte cell system. It is possible that the increase in JAK2 gene transcription occurred as a response to a transient decrease in total amount of JAK2 tyrosine phosphorylation induced by endotoxin. Alternatively, the rapid increase in JAK2 mRNA may be indicative of regulatory sequences in the JAK2 promoter that are activated in response to endotoxin or endotoxin-induced proinflammatory cytokines. In future studies, it will be important to investigate the mechanism and physiological importance of JAK2 mRNA up-regulation in catabolic disease states.

GH receptor abundance in the liver is known to be sensitive to nutrient regulation (19), and recent studies have shown that GH receptor mRNA is reduced after elective surgery (20). As a potential mechanism to explain the effects of surgical stress on GH receptor expression, studies in cultured rat hepatocytes have demonstrated decreased GH receptor mRNA levels after incubation with tumor necrosis factor-α or interleukin-1 (21, 46). Because endotoxin stimulates the synthesis of tumor necrosis factor-α, interleukin-1, and other cytokines in vivo, it was important to investigate the possibility that the altered stoichiometry of JAK2 phosphorylation and decreased STAT5 tyrosine phosphorylation induced by endotoxin might reflect a change in the amount of GH receptor in the liver. Using a specific GH receptor antibody preparation, GH receptor levels were shown by immunoprecipitation and immunoblotting to be unchanged 4 h after the administration of endotoxin. The endotoxin-induced alterations in JAK2 and STAT5 were maximal at this time point, and it therefore can be concluded that they occur independent of changes in the abundance of GH receptors in the liver. In contrast to our finding of unaltered GH receptor abundance 4 h after endotoxin, in a recent publication by Defalque et al. (47), a decreased number of liver GH receptors was observed 5 h after endotoxin treatment. This difference may have occurred because of the use of younger animals (3-week-old females vs. approximately 8-week-old males) as well as higher doses of endotoxin (2.5- and 7.5-fold higher) in the Defalque study.

As GH was infused at a high dose that insured saturation of available GH receptors, our data provide strong evidence for postreceptor resistance in the GH signaling pathway. Although it has been suggested that GH resistance in catabolic states may result from a combination of decreased receptor abundance and inhibited postreceptor signaling, these data represent the first demonstration of GH resistance occurring in the absence of a change in receptor abundance. With longer term endotoxin exposure or other sustained stress states that influence nutritional status as well as stimulating inflammatory cytokines, GH receptor levels would be expected to decrease, and compromised GH actions in the liver and other tissues then would result from a combination of decreased receptor abundance and postreceptor GH resistance. Because significant GH effects on hepatic IGF-I mRNA levels do not occur until approximately 24 h after GH administration in normal, nonhypophysectomized rats (data not shown), determinations of such downstream responses were not included in this study of acute GH signaling responses.

As candidate mediators of postreceptor GH resistance, we determined the effects of endotoxin on hepatic mRNAs for members of the recently described SOCS gene family (29, 31, 32, 34). The levels of mRNA for several SOCS genes have been shown to rapidly increase in the liver and other cell types after cytokine receptor activation (29, 35, 36), and the SOCS proteins are hypothesized to function as negative feedback regulators of cytokine signaling (35). We focused on three members of the SOCS gene family (CIS, SOCS-2, and SOCS-3) that are relatively abundant in the liver and responsive to cytokine regulation. As rat cDNAs corresponding to these proteins were not available, we used RNA PCR methods to obtain clones from rat liver and established their full coding sequences. This both provided cDNA probes with assured homology for use in Northern blotting of rat tissues and defined for the first time the sequences of these proteins in the rat. Northern blotting analysis of liver total RNA preparations demonstrated a rapid 4-fold increase in CIS and a 10-fold increase in SOCS-3 mRNA, but only a small, transient increase in SOCS-2 mRNA after the administration of a single dose of endotoxin. SOCS-3 mRNA was not only most markedly affected by endotoxin, but it also remained significantly elevated for at least 6 h after endotoxin administration.

The increases in mRNAs for the SOCS genes correlate temporally with endotoxin-induced resistance to GH-stimulated STAT5 and JAK2 tyrosine phosphorylation, consistent with a role for the SOCS proteins in mediating the effects of endotoxin on GH signaling. In CHO cells cotransfected with an Spi 2.1-CAT reporter construct and the rat GH receptor, overexpression of SOCS-3 and the closely related SOCS-1 protein inhibited GH *trans*-activation of the Spi 2.1 promoter (35), indicating a capacity of these proteins to negatively regulate GH signaling. By contrast, CIS did not alter GH induction of the Spi 2.1 CAT reporter, and SOCS-2 augmented the effect of GH. Although additional studies are needed in more physiological systems, these findings suggest that specific SOCS gene products may have distinct effects on the actions of GH. SOCS-1 has been shown to directly interact with JAK2 as well as other JAK tyrosine phosphorylation sites.
kinases (32, 34), resulting in inhibition of JAK tyrosine kinase activity and JAK-catalyzed tyrosine phosphorylation of STAT proteins (34). It has been suggested that a conserved sequence in the C-terminal region of SOCS-1, which is similar to the autophosphorylation site that regulates JAK tyrosine kinase activity, may interact with the catalytic region of JAK kinases and thus inhibit their activity. Although not yet investigated, similar binding of SOCS-3 to JAK2 could explain the decreased stoichiometry of JAK2 phosphorylation and inhibited STAT5 phosphorylation induced by endotoxin in this study.

GH itself has been shown to induce mRNAs for SOCS-3 and, to a lesser extent, SOCS-1, in mouse liver (35). It is likely that GH-induced SOCS protein expression functions in a negative feedback loop to attenuate GH signaling, as has been suggested for interleukins and other cytokines. Interleukin-6 infusion in mice also leads to a rapid increase in hepatic SOCS mRNAs, including SOCS-1, SOCS-2, SOCS-3, and CIS (29). In bone marrow cells, a broad spectrum of cytokines induces SOCS mRNAs (29), and it is probable that multiple cytokines also regulate hepatic SOCS gene expression.

After endotoxin administration, multiple cytokines are elaborated (2), each of which may act in the liver to induce increased transcription of SOCS family mRNAs. We propose that the marked increase in SOCS-3 and other SOCS proteins induced by endotoxin could result not only in feedback inhibition of cytokine signaling, but also in resistance to GH. A phenomenon of specificity-spillover has been described (48), in which high levels of a hormone can result in its binding to receptors for another hormone in addition to its own receptors (e.g. GH binding to PRL as well as GH receptors in patients with acromegaly) (48, 49). Our data suggest that a postreceptor specificity-spillover mechanism involving the inhibition of GH signaling by cytokine-induced SOCS proteins may contribute to the development of GH resistance in catabolic states.

In a similar manner, endotoxin-induced SOCS genes may have clinically important inhibitory effects on other cytokine signaling pathways.

Further investigation of the proposed role of SOCS proteins in GH resistance will require determination of the levels of these proteins and their interactions with GH signaling intermediates in rat liver. Although antibodies to several SOCS proteins can be obtained from commercial suppliers, our studies of multiple currently available antibody preparations have demonstrated their interaction with recombiant SOCS proteins at high concentrations, but not with endogenous SOCS proteins in rat tissues. In addition to studies on the CIS, SOCS-2, and SOCS-3 proteins, it will be important to investigate the role of SOCS-1 in endotoxin-induced GH resistance.

In summary, endotoxin infusion results in a time-dependent increase in GH-stimulated tyrosine phosphorylation of STAT5 in the liver that is not explained by decreased tissue content of the STAT5 protein. This is associated with an increase in Jak2 protein content and a 50% decrease in the amount of GH-stimulated phosphotyrosine per Jak2 in endotoxin-treated animals, which is preceded by a marked increase in Jak2 mRNA. The GH signaling changes after endotoxin infusion are not explained by diminished GH receptor abundance in the liver. SOCS mRNAs, including CIS, SOCS-2, and SOCS-3, are rapidly up-regulated after endotoxin administration, suggesting the involvement of SOCS proteins in endotoxin-induced alterations in the GH signaling pathway.

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