Role of the Suppressor of Cytokine Signaling-3 in Mediating the Inhibitory Effects of Interleukin-1β on the Growth Hormone-dependent Transcription of the Acid-labile Subunit Gene in Liver Cells

(Received for publication, September 1, 1999, and in revised form, November 4, 1999)

Yves R. Boisclair‡§, Jianrong Wang‡, Jiarong Shi‡, Kelley R. Hurst‡, and Guck T. Ooi¶

From the ‡Department of Animal Science, Cornell University, Ithaca, New York 14853 and ¶Prince Henry’s Institute of Medical Research, Clayton, Victoria 3168, Australia

During catabolic diseases such as sepsis, inflammation, and infection, a state of growth hormone (GH) resistance develops in liver. This has been attributed in part to increased production of the proinflammatory cytokine interleukin-1β (IL-1β). To determine how IL-1β induces GH resistance, we studied the acid-labile subunit (ALS) gene whose hepatic transcription is increased by GH via the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway. IL-1β reduced the ability of GH to stimulate ALS mRNA in rat primary hepatocytes and ALS promoter activity in H4-II-E rat hepatoma cells. This inhibition was dependent on ALS-GAS1, an element resembling a γ-interferon activated sequence that mediates the transcriptional effects of GH. Inhibition by IL-1β was also associated with a reduction of GH-dependent binding of STAT5 to this element after chronic (8 and 24 h), but not after acute treatment (15 min). Because these results indicated that the inhibition by IL-1β was indirect, expression of the recently discovered suppressors of cytokine action (SOCS) was examined in liver cells. IL-1β did not alter the expression of SOCS1, SOCS2, and CIS, indicating that they are not involved. In contrast, IL-1β increased SOCS3 mRNA by 8-fold after 24 h of treatment, whereas GH had no effect. Forced expression of SOCS3 was just as effective as IL-1β in reducing the GH induction of ALS promoter activity in H4-II-E rat hepatoma cells. Similar results were observed in primary rat hepatocytes. We conclude that the induction of SOCS3 by IL-1β contributes to the development of GH resistance in liver, and represents a mechanism by which cytokines such as IL-1β cross-talk with cytokines using the JAK-STAT pathway.

Many of the anabolic actions of growth hormone (GH) are mediated by insulin-like growth factor (IGF)-1 (1–4). In postnatal animals, most of IGF-I circulates in ternary complexes of 150 kDa composed of one molecule each of IGF-I, IGF-binding protein-3, or IGF-binding protein-5, and an acid-labile subunit (ALS) (3, 5, 6). Ternary complexes have an extended half-life and represent a reservoir of bioactive IGF-I in the circulation (3, 6–8). They are almost completely absent in the plasma of GH-deficient rodents because circulating ALS and IGF-I originates mostly from the liver where transcription of both genes is dependent on adequate circulating levels of GH (9–11).

The effects of GH on hepatic gene transcription are impaired in many diseased states, including malnutrition, infection, inflammation, and sepsis (12, 13). Individuals suffering from these diseased states have low circulating levels of IGF-I in the presence of normal or elevated concentrations of GH, and are unable to increase circulating IGF-I in response to GH therapy (13, 14). Deleterious consequences of reduced circulating IGF-I include the development of negative N balance and muscle wasting (12, 15). Increased production of proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α, and interleukin-6 by monocytes and macrophages have been implicated in the development of a GH-resistant state in some of these catabolic diseases (12). In rats, administration of the endotoxin lipopolysaccharide, an inducer of IL-1β and tumor necrosis factor-α, or direct administration of these cytokines reduced hepatic production and circulating levels of IGF-I as well as the ability of GH to increase hepatic IGF-I mRNA (16–19). The molecular mechanism by which these cytokines induces a state of GH resistance in liver has not been completely elucidated. They could repress transcription of GH-responsive genes directly, or they could prevent transmission of the GH signal. This last mechanism is suggested by the ability of IL-1β to block the GH-dependent induction of ALS and IGF-I mRNA in primary hepatocytes (20–22).

In the case of IGF-I, studies of the mechanism underlying the effect of IL-1β are complicated by the lack of information regarding regulatory sequence and transcription factors involved in GH stimulation of gene transcription (23, 24). In contrast, we have demonstrated that GH stimulates the transcription of the ALS gene in primary hepatocytes and in the H4-II-E hepatoma liver cell line by inducing the binding of signal transducer and activator of transcription (STAT)-5a and -5b to a single element resembling a γ-interferon activated sequence (GAS) in the promoter (25). Using this model of GH-regulated transcription in liver, we demonstrate that IL-1β inhibits the GH-induced transcription of the ALS gene by inducing the suppressor of cytokine signaling-3 (SOCS3), a member of a family of intracellular protein involved in the termination of signaling by the JAK-STAT pathway (26, 27). These findings suggest that SOCS can...
play broader roles such as mediating cross-talk between JAK-STAT and unrelated signal transduction pathways.

EXPERIMENTAL PROCEDURES

General Reagents—Restriction endonucleases, DNA polymerase, and DNA modifying enzymes were purchased from New England Biolabs, Inc. (Beverly, MA). Tissue culture media and bovine insulin were from Life Technologies, Inc., and protease inhibitors and dexamethasone from Sigma. The basement membrane Matrigel® was purchased from Becton Dickinson Labware (Bedford, MA). Recombinant bovine GH was a gift from Provia (St. Louis, MO), recombinant human IL-1β a donation from the Biological Resources Branch of the National Cancer Institute (Frederick, MD). DEAE-dextran and the DNA alternating copolymers poly(dA-dT)poly(dA-dT) were purchased from Amersham Pharmacia Biotech. Oligonucleotides were custom-made by Life Technologies, Inc. or by the BioResource Center at Cornell University. Radionucleotides were obtained from NEN Life Science Products.

Plasmids—Construction of the mouse ALS promoter plasmids has been described in detail (25). Briefly, fragments corresponding to nt −703 to nt −11 (A..TGT) or to nt −1627 to nt −11 of the mouse ALS gene were amplified by polymerase chain reaction and inserted between the KpnI and HindIII restriction sites of the promoterless luciferase vector pGL3-Basic (Promega Corp.). Recombinant bovine GH was a gift from Provia (St. Louis, MO), recombinant human IL-1β a donation from the Biological Resources Branch of the National Cancer Institute (Frederick, MD). DEAE-dextran and the DNA alternating copolymers poly(dA-dT)poly(dA-dT) were purchased from Amersham Pharmacia Biotech. Oligonucleotides were custom-made by Life Technologies, Inc. or by the BioResource Center at Cornell University. Radionucleotides were obtained from NEN Life Science Products.

Construction of the mouse ALS promoter plasmids has been described in detail (25). Briefly, fragments corresponding to nt −703 to nt −11 (A..TGT) or to nt −1627 to nt −11 of the mouse ALS gene were amplified by polymerase chain reaction and inserted between the KpnI and HindIII restriction sites of the promoterless luciferase vector pGL3-Basic (Promega Corp.). Recombinant bovine GH was a gift from Provia (St. Louis, MO), recombinant human IL-1β a donation from the Biological Resources Branch of the National Cancer Institute (Frederick, MD). DEAE-dextran and the DNA alternating copolymers poly(dA-dT)poly(dA-dT) were purchased from Amersham Pharmacia Biotech. Oligonucleotides were custom-made by Life Technologies, Inc. or by the BioResource Center at Cornell University. Radionucleotides were obtained from NEN Life Science Products.

Fidelity Vent polymerase, and used to prepare duplicate plasmids. The independent polymerase chain reactions were performed with the high fidelity Vent polymerase, and used to prepare duplicate plasmids. The expression vector pEF-FLAG-I/SOCS3 was constructed by inserting the fidelity Vent polymerase, and used to prepare duplicate plasmids. The independent polymerase chain reactions were performed with the high fidelity Vent polymerase, and used to prepare duplicate plasmids. The expression vector pEF-FLAG-I/SOCS3 was constructed by inserting the coding region of the mouse SOCS3 cDNA into the mammalian expression vector pEF-FLAG-I, and was obtained from Drs. D. J. Hilton and R. Starr (Walter and Eliza Hall Institute of Medical Research, Parkville, Australia) (28). All plasmids were purified by ion-exchange chromatography (Qiagen, Chatsworth, CA).

Culture of Rat Liver Cells—H4-II-E cells were plated at a density of 1.0 × 10⁶ cells/9.5 cm² in Primaria culture dishes (Becton Dickinson Labware) (29). The basement membrane Matrigel® was purchased from Becton Dickinson Labware (Bedford, MA). Recombinant bovine GH was a gift from Provia (St. Louis, MO), recombinant human IL-1β a donation from the Biological Resources Branch of the National Cancer Institute (Frederick, MD). DEAE-dextran and the DNA alternating copolymers poly(dA-dT)poly(dA-dT) were purchased from Amersham Pharmacia Biotech. Oligonucleotides were custom-made by Life Technologies, Inc. or by the BioResource Center at Cornell University. Radionucleotides were obtained from NEN Life Science Products.

Construction of the mouse ALS promoter plasmids has been described in detail (25). Briefly, fragments corresponding to nt −703 to nt −11 (A..TGT) or to nt −1627 to nt −11 of the mouse ALS gene were amplified by polymerase chain reaction and inserted between the KpnI and HindIII restriction sites of the promoterless luciferase vector pGL3-Basic (Promega Corp.). Recombinant bovine GH was a gift from Provia (St. Louis, MO), recombinant human IL-1β a donation from the Biological Resources Branch of the National Cancer Institute (Frederick, MD). DEAE-dextran and the DNA alternating copolymers poly(dA-dT)poly(dA-dT) were purchased from Amersham Pharmacia Biotech. Oligonucleotides were custom-made by Life Technologies, Inc. or by the BioResource Center at Cornell University. Radionucleotides were obtained from NEN Life Science Products.

Primary hepatocytes were isolated from adult male rats by the recirculating collagenase perfusion method (25), according to procedures approved by the Cornell University Institutional Animal Care and Use Committee. They were plated at a density of 1.0 × 10⁶ cells/9.5 cm² in Primaria culture dishes (Becton Dickinson Labware) and allowed to attach for 5 h in modified William’s E medium (MWEM) containing 10% fetal calf serum (FCS). The primary hepatocytes were washed three times with phosphate-buffered saline, and incubated for 16 h in serum-free medium. Media were then changed to fresh serum-free medium (MWEM for H4-II-E, MWEM containing 500 μg/ml Matrigel® for the primary hepatocytes) supplemented with various cytokines as indicated in the figure legends. NORTHERN ANALYSIS—Total RNA was prepared from rat liver cells by the acid guanidium thiocyanate phenol-chloroform method, and quantified by absorbance at 260 nm (11). Total RNA (15 μg/lane) was electrophoresed on a 1.2% agarose/formaldehyde gel, blotted onto a nylon membrane, and hybridized to [α-32P]dCTP-labeled DNA probes. Probes used included the coding region of mouse SOCS1, SOCS2, SOCS3, and CIS cDNA (obtained from Drs. Hilton and Starr; Ref. 28), and a DNA fragment corresponding to nt +1262 to nt +1555 of the rat ALS cDNA (A..TGT) (29). Staining with ethidium bromide confirmed that ribosomal RNA was intact and that equal amounts of RNA were loaded in each lane. The relative abundance of each signal was quantified by phosphorimaging using a Fuji BAS 1000 unit (Fuji Medical Systems, Stamford, CT).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay Assays—Nuclear extracts were prepared from rat liver cells by the mini-extraction procedure of Lee et al. (30), modified by the inclusion of protease and phosphatase inhibitors in the various buffers (25). Protein concentration of each extract was determined by the Lowry method.

Nuclear extracts (6 μg) were preincubated for 10 min in a buffer containing 1 μg of poly(dA-dT)poly(dA-dT), 20 μm Hepes, pH 7.9, 10% glycerol, 50 μm NaCl, 1 μm MgCl₂, 1 mM EDTA, and 1 μm dithiothreitol (25). Then a radiolabeled probe (4–9 fmol, 20,000–40,000 cpm), corresponding to nt −638 to nt −621 of the mouse ALS gene (ALS2), was added to this solution. After 20 min at room temperature, protein–DNA complexes were separated on a 5% non-denaturing polyacrylamide gel (38.1, acrylamide: bisacrylamide, 2% glycerol, 22 mTorr, 0.5 mm EDTA; pH 8.3) at 15 mA (2 h at 4 °C). Gels were dried and autoradiographed at −70 °C using intensifying screens. Relative intensity of the specific protein–DNA complexes were quantified by phosphorimaging.

Transfection of Rat Liver Cells—Transfections were performed exactly as described previously (25), except that cells were grown in six-well plates. Briefly, each well of near confluent H4-II-E cells was electroporated with 0.5 μg of a DNA solution (0.5 μg DEAE-dextran, 0.3 μg of firefly luciferase plasmid, 0.3 μg of plasmid pRL-TK, and, when indicated, 0.5 μg of pEF-FLAG-ISOC3S or pEF-FLAG-I). The plasmid pRL-TK (Promega) encodes Renilla luciferase and was used to correct for variation in transfection efficiency. After a 40-h recovery period in DMEM supplemented with 10% fetal calf serum, media were changed to serum-free DMEM supplemented with cytokines as indicated in the figure legends. Twenty hours later, cell lysates were assayed for firefly and Renilla luciferase by the Dual-Luciferase Reporter System (Promega).

For primary hepatocytes, each well of a six-well plate was transfected for 14 h with a 1 ml solution of serum-free MWEM containing 1.2 μg of the firefly luciferase plasmid, 300 ng of pRL-TK, and 15 μg of Lipofectin (Life Technologies, Inc.). In some experiments, 0.5 μg of pEF-FLAG-I/SOCS3 or pEF-FLAG-I was added to this solution. After transfection, the cells were cultured for 48 h in MWEM (supplemented with 500 μg/ml Matrigel® for the first 24 h) in the absence or presence of cytokines as specified in the figure legends. Firefly and Renilla luciferase were then measured as described above.

RESULTS

IL-1β Inhibits the GH-dependent Induction of ALS mRNA in Primary Hepatocytes and of ALS Promoter Activity in H4-II-E Cells—IL-1β has been reported to reduce the ability of GH to increase the synthesis of ALS in primary rat hepatocytes (20). To document this effect, primary hepatocytes were incubated for 24 h in the absence or in the presence of a maximally effective dose of 100 ng/ml GH with increasing concentrations of IL-1β (Fig. 1). GH increased the abundance of ALS mRNA by 12-fold, whereas IL-1β inhibited this stimulation in a dose-dependent manner. Inhibition by IL-1β reached a maximum at the dose of 10 ng/ml (20).

We next determine whether the inhibitory effects of IL-1β occurred at the level of transcription. The H4-II-E rat hepatoma cells, which we have shown to recapitulate exactly the mechanisms leading to basal and GH-stimulated transcription of the mouse ALS gene (11, 25), were transiently transfected with a luciferase construct driven by a promoter fragment corresponding to nt −703 to nt −11 (703WT) of the mouse ALS mRNA, was detected. Each lane represents RNA from a single culture dish. Similar results were obtained in a duplicate experiment.

![Figure 1. IL-1β inhibits the stimulation effects of GH on the abundance of ALS mRNA in a dose-dependent manner in primary rat hepatocytes.](image-url)
gene. Transfected cells were incubated for 24 h in the absence or in the presence of 100 ng/ml GH with increasing concentrations of IL-1β (Fig. 2). As shown previously (25), GH stimulated the luciferase activity of this construct by 2.6-fold. This stimulation was suppressed by IL-1β in a dose-dependent manner with a maximal inhibition of 43% at the dose of 10 ng/ml, similar to the concentration required for maximal inhibition of ALS mRNA in primary rat hepatocytes. These results indicate that inhibition of transcription is an important mechanism by which IL-1β reduces the GH-mediated increase in ALS gene expression.

**The Inhibitory Effect of IL-1β Is Dependent on the GH-responsive GAS Element**—We have shown previously that ALSGAS1, a single GAS element located between nt –633 and nt –545, mediates the GH stimulation of ALS gene transcription (25). Thus, the inhibitory effect of IL-1β could be dependent on the GAS element or could involve a distinct regulatory sequence in the ALS promoter. To distinguish between these possibilities, H4-II-E cells were transfected with the luciferase plasmids 703WT, 703ΔGAS1, or 703ΔGAS2, and treated with GH with or without IL-1β. Plasmid 703ΔGAS1 is identical to 703WT, except for an inactivating block mutation of the GH-responsive element ALSGAS1. Plasmid 703ΔGAS2 contains an identical mutation in a GAS-like sequence located between nt –553 and –545 that plays no role in the GH stimulation of the ALS promoter (25).

IL-1β alone did not alter the basal activity of the wild type or of the mutant luciferase constructs (Fig. 3). Similar results were obtained with the luciferase plasmid 1627WT, which contains an additional 925 base pairs of 5’-flanking sequence from the mouse ALS promoter (results not shown). These results indicate that the promoter region comprised between nt –1627 and nt –11 does not contain an IL-1β-responsive element.

In contrast, IL-1β readily inhibited the GH-dependent increase of luciferase activity in H4-II-E cells transfected with the plasmids 703WT or 703ΔGAS2. This inhibition was not observed in H4-II-E cells transfected with the plasmid 703ΔGAS1, which harbors a mutation of the GH-responsive element, ALSGAS1. Therefore, inhibition of ALS expression by IL-1β is dependent on GH stimulation and does not occur in GH-treated cells unless the GH response element of the ALS promoter is present.

**IL-1β Decreases the GH-dependent Binding of STAT5 to the ALSGAS1 Element**—GH stimulates the transcription of the ALS gene through activation of STAT5a and STAT5b, followed by their binding to the ALSGAS1 element (25). Tyrosine phosphorylation of STAT5 by receptor-associated JAK2 kinases is required for nuclear translocation and DNA binding (31). To determine whether formation of the GH-dependent STAT5-DNA complex is affected by IL-1β, nuclear extracts were prepared from H4-II-E cells treated for 15 min to 24 h with GH, IL-1β or the combination of both cytokines, and analyzed by EMSA using a radiolabeled ALSGAS1 probe (Fig. 4).

As expected, GH was able to induce the binding of STAT5 isoforms to ALSGAS12 (Fig. 4). Binding was maximal after 15 min of treatment and remained obvious after 24 h of continuous incubation with GH. In contrast, nuclear extracts from IL-1β-treated cells did not induce the formation of any protein-DNA complexes over this time period. However, when used in conjunction with GH, IL-1β was able to reduce the abundance of the GH-dependent STAT5-ALS complex after 8 and 24 h of incubation (Fig. 4, compare lanes 8 and 10, and lanes 11 and 13), but had no effects at earlier times (15 min and 1 h; compare lanes 2 and 4, and lanes 5 and 7). The abundance of total STAT5, however, was not reduced by incubation with IL-1β with or without GH over this time period when analyzed by immunoblotting (results not shown). Therefore, these results indicate that IL-1β reduces the abundance of activated STAT5 binding to the ALSGAS1 element, leading to a decrease in the GH-dependent transcription of the ALS gene. The inhibitory effect of IL-1β on the GH transactivation of the ALS promoter requires a functional GAS-like element. 703WT corresponds to the firefly luciferase plasmid pGL3 basic containing nt –703 to nt –11 of the mouse ALS promoter. The nt –703 to nt –11 promoter fragment contains two GAS-like sequences located between nt –633 and nt –625 (ALSGAS1) and between nt –553 and nt –545 (ALSGAS2). Luciferase plasmids containing individual block substitution mutants were obtained by replacing ALSGAS1 (703ΔGAS1) or ALSGAS2 (703ΔGAS2) by an EcoRI linker. Firefly luciferase plasmids (0.7 μg) and the plasmid pTK-Renilla (300 ng) were transfected in duplicate into H4-II-E cells by the DEAE-dextran method. The transfected cells were incubated for 24 h in serum-free medium in the absence or in the presence of GH (100 ng/ml), IL-1β (10 ng/ml) or the combination of both cytokines (GH+IL-1β). Firefly luciferase activity was measured in cell extracts and corrected for Renilla luciferase activity. The fold stimulation (mean ± S.E. of three experiments) was calculated as the ratio of firefly luciferase activity in the presence and in the absence of cytokine. Bars with different letters differ at p < 0.05 using one-way ANOVA, followed by Fisher protected least significant differences analysis.

6 We have shown previously that this complex is specific in H4-II-E cells and in primary hepatocytes as shown by competition with excess ALSGAS1 oligonucleotide, but not by an excess of unrelated oligonucleotide (25). We also showed, using specific antibodies, that this complex is composed exclusively of STAT5a and STAT5b (25).
inhibitory effects of IL-1β on STAT5 binding develops slowly, suggesting that they might represent secondary effects.

Role of SOCS in Mediating the Inhibitory Effects of IL-1β on the GH-dependent Induction of ALS Promoter Activity in H4-II-E Cells—Recently, a family of proteins able to suppress signaling by the JAK-STAT pathway was discovered (26, 27). These proteins, called suppressors of cytokine signaling (SOCS), inhibit transcriptional responses to many cytokines, in part by reducing the abundance of activated STAT (32–37). To determine the possible involvement of SOCS in mediating the inhibitory effects of IL-1β on ALS promoter activity, H4-II-E cells were treated with maximally effective concentrations of GH, IL-1β or the combination of both. Total RNA was prepared before (15 min) and after (8 and 24 h) the suppression by IL-1β of the GH-dependent formation of STAT5-ALSGAS1 complexes, and analyzed by Northern blotting for the steady state abundance of SOCS1, SOCS2, SOCS3, and CIS mRNAs (Fig. 5).

At the 15-min time point, abundance of CIS and SOCS2 mRNAs was low and unaffected by the various treatments. At 8 and 24 h, GH with or without IL-1β caused similar induction of SOCS2 and CIS mRNA levels, indicating GH-dependent regulation at these times. At the 24-h time point, SOCS2 mRNA was repressed by IL-1β. Finally, the abundance of SOCS1 mRNA remained very low and unresponsive to the various cytokine treatments over the study period. Overall, these changes in SOCS1, SOCS2, and CIS mRNAs cannot account for the inhibitory effects of IL-1β on GH stimulation of ALS gene expression.

In contrast, the abundance of SOCS3 mRNA was increased by 2- and 8-fold following incubation of H4-II-E cells with IL-1β for 8 and 24 h, respectively (p < 0.05). A similar stimulation was seen in cells treated with IL-1β in the presence of GH, indicating that it was dependent on IL-1β but independent of GH. Induction of SOCS3 mRNA by IL-1β, therefore, correlates temporally with the inhibition of the GH-dependent events responsible for increased ALS gene expression (i.e. GH-dependent binding of STAT5 to ALSGAS1 at 8 and 24 h, and GH stimulation of ALS promoter activity at 24 h). These findings suggest that SOCS3 plays a role in mediating the inhibitory effects of IL-1β.

To evaluate directly the ability of SOCS3 to inhibit the action of GH, H4-II-E cells were transfected with the luciferase plasmids 703WT or 703ΔGAS1, either in the absence or in the presence of the mouse SOCS3 expression vector or the corresponding empty vector. Transfected cells were then incubated with or without GH for 24 h (Fig. 6). Overexpression of SOCS3 reduced the ability of GH to increase luciferase activity by 50%. This inhibitory effect is specific to the GH-responsive GAS element, as overexpression of SOCS3 was not able to suppress luciferase activity in GH-treated H4-II-E cells transfected with the plasmid 703ΔGAS1, the promoter construct containing a block mutation of the ALSGAS1 element.

SOCS3 Is Also Induced by IL-1β in Primary Hepatocytes and Inhibits the GH-dependent Stimulation of mALS Promoter Activity—Finally, we determined whether a similar mechanism underlies the inhibition by IL-1β of the GH-dependent induction of ALS mRNA in isolated primary liver cells. In primary hepatocytes transfected with the GH-responsive ALS plasmid 703WT, IL-1β repressed ALS promoter activity only in the presence of GH (Fig. 7). This repression was also associated with a decrease in the formation of the GH-dependent STAT5-
ALSGAS1 complex after 8 h of treatment with IL-1β, but not after 15 min (Fig. 7). Therefore, similar to that shown in H4-II-E cells, inhibition of the GH-activation of ALS gene expression by IL-1β is at the level of transcription in isolated liver cells and occurs after a few hours of treatment.

To determine if SOCS could mediate this inhibition, we compared time-dependent changes of ALS and SOCS mRNA after the addition of IL-1β to GH-treated primary hepatocytes. Primary hepatocytes were first incubated for 24 h with GH to increase steady state levels of ALS mRNA, followed by a second 24-h period with GH in the absence or in the presence of IL-1β; total RNA was prepared at various times during the second 24-h period. The inhibitory effect of IL-1β on the GH-stimulated increase of ALS mRNA was not detected after 4 h of incubation, but was apparent after 8 and 24 h of incubation (Fig. 8). Levels of SOCS2 mRNA and CIS mRNA were very similar with and without IL-1β at all times examined, whereas SOCS1 mRNA was not detected (Fig. 8 and results not shown). In contrast, IL-1β caused a 4-fold increase in SOCS3 mRNA after 4 h of treatment, an induction that was maintained at later times. This induction of SOCS3 is consistent with the inhibition of GH-responses by IL-1β (i.e. reduction of ALS mRNA at 8 and 24 h, and reduction of STAT5-ALSGAS1 abundance after 8 h of incubation with IL-1β).

Finally, we determined whether SOCS3 could inhibit the GH-mediated increase of mALS promoter activity in primary hepatocytes. Overexpression of SOCS3 caused a 52% reduction in the GH-stimulated increase in luciferase activity when transfected with the GH-responsive ALS plasmid 703WT (Fig. 8). Overall, these results indicate that the induction of SOCS3 by IL-1β leads to a reduction in the binding of STAT5 to the ALSGAS1 element, and subsequently in the GH-dependent transcription of the ALS gene.

DISCUSSION

Formation of the 150-kDa IGF-binding protein complex in adult serum requires a functional GH axis (3, 6, 9). This reflects, in part, the GH stimulation of ALS gene transcription in liver, resulting in increased concentration of circulating ALS (11). We recently showed that this transcriptional activation is mediated by the binding of STAT5 isoforms to ALSGAS1, a GAS-like element located in the proximal promoter (25). In addition, ALS synthesis is negatively regulated by the inflammatory cytokine IL-1α, but the mechanisms underlying this inhibitory effect have not been elucidated (20). Here, we demonstrate that a major portion of the negative effects of IL-1β on ALS mRNA in rat liver cells represents a modulation of the GH signaling pathway, and implicate SOCS3 as possible mediator of this inhibition.

H4-II-E cells have been used to study the transcriptional responses of hepatic genes to many hormones and cytokines (38, 39). When transiently transfected with mALS promoter constructs, they are a valid model to study the transcriptional regulation of the gene (11, 25). In these cells, IL-1β did not repress transcription of the proximal mALS promoter, even though IL-1β can regulate gene expression directly via transcription factors such as AP1 and NF-κB (40, 41). This result does not rule out a transcriptional effects of IL-1β on the ALS gene, but implies that a direct action, if it exists, must be mediated by a response element located outside the nt −1627 and nt −11 promoter region. Instead, we show that repression occurred only in the presence of GH, and was dependent on the presence of ALSGAS1, the GH response element of this promoter (25). Repression by IL-1β correlated temporally with a reduction in the formation of the GH-dependent STAT5-ALSGAS1 complex. These results indicate that a major portion of the effects of IL-1β is to antagonize the GH signal transduced by STAT5.

Recently, SOCS1 was cloned from its ability to suppress the responses of monocytic leukemic M1 cells to IL-6 and, independently, from structural properties (i.e., interaction with JAK2 or homology with the SH2 domain of STAT; Refs. 28, 42, and 43). Search of data base for homologous sequences led to the identification of 6 additional members of this family (SOCS2–7 and CIS) (26, 27). They are induced rapidly by cytokines, which use the JAK-STAT pathway, and confer resistance to these cytokines, in part by inhibiting the activation of STATs (32–37). Surprisingly, IL-1β, a cytokine that signals via kinases such as NF-κB-inducing kinase and c-Jun N-terminal kinase (40, 41), was also shown to increase the expression of SOCS2, SOCS3, and CIS in bone marrow of mice (28), suggesting to us that SOCS could mediate the GH resistance induced by IL-1β. Our data provide evidence in support of this hypothesis. First, SOCS3 is induced by IL-1β in H4-II-E cells; this induction has a slow onset compared with the induction described for cytokine signaling via the JAK-STAT pathway (28, 33, 36, 37, 44), but occurs at the times when signs of GH resistance developed in IL-1β-treated cells (i.e. decreased activation of STAT5 after 8 and 24 h of treatment and decreased inhibition of the GH signal transduced by STAT5).

FIG. 7. IL-1β inhibits transmission of the GH signal in primary rat hepatocytes. Left panel, IL-1β inhibits the GH-dependent induction of the mALS promoter. The mouse luciferase plasmid 703WT was cotransfected (1.2 μg) with plasmid pTK-Renilla (300 ng) in triplicate into primary hepatocytes using Lipofectin. Transfected cells were treated for 48 h in the absence or in the presence of 100 ng/ml GH or 10 ng/ml IL-1β, alone or in combination. Luciferase activity was measured in cell lysates and corrected for Renilla luciferase activity. The fold stimulation (mean ± S.E. of 2 experiments) was calculated as the ratio of luciferase in the presence and in the absence of cytokine. Bars with different letters differ at p < 0.05 using one-way ANOVA followed by Fisher protected least significant differences analysis. Right panel, IL-1β reduces the GH-dependent binding of STAT5 to ALSGAS1. Nuclear extracts were prepared from primary hepatocytes cultivated in serum-free medium for 16 h, followed by a 15-min or 8-h period of incubation in the absence (−) or presence (+) of GH (100 ng/ml), IL-1β (10 ng/ml), or the combination of both cytokines (GH+IL-1β). They were incubated with labeled oligonucleotides (20,000 cpm) corresponding to the ALSGAS1 element of the mouse ALS gene, and EMSA was performed as before. In order to visualize clearly the ALSGAS1 complex at the 8-h period of incubation, the exposure time of the autoradiogram was twice as long as the exposure time used for the 15-min period. Position of the specific ALSGAS1 complex is indicated by an arrow (see Footnote 2).
Role of SOCS3 in IL-1β Inhibition of GH Action in Liver

FIG. 8. SOCS3 is induced by IL-1β and decreases the GH activation of the mALS promoter in primary rat hepatocytes. Left panel, IL-1β inhibition of ALS mRNA in the presence of GH is associated with increased expression of SOCS3. Hepatocytes were cultivated for 16 h in MWM serum-free medium, followed by a 24-h period in the presence of 100 ng/ml GH. Then, medium containing only 100 ng/ml GH (GH) or the combination of 100 ng/ml GH and 10 ng/ml IL-1β (GH + IL-1β) was added. Total RNA was isolated at various times (4, 8, or 24 h) after the addition of IL-1β, and the mRNA abundance for ALS and various SOCS was determined by Northern analysis. Each lane represents RNA from a single culture dish. Similar results were obtained in a duplicate experiment. Right panel, forced expression of SOCS3 reduces the GH-dependent activation of the mALS promoter. The mouse luciferase plasmid 703WT was cotransfected (1.2 μg) with plasmid pTK-Renilla (300 ng) either in the absence (-) or in the presence (+) of the mouse SOCS3 expression vector (SOCS3) or the corresponding empty vector (Empty) (0.5 μg) in triplicate into primary hepatocytes using Lipofectin. Transfected hepatocytes were treated for 48 h in the absence or in the presence of 100 ng/ml GH. Luciferase activity was measured in cell lysates and corrected to the Renilla luciferase activity. The -fold stimulation (mean ± S.E. of two experiments) was calculated as the ratio of luciferase activity in the presence and in the absence of GH. Bars with different letters differ at p < 0.05 using one-way ANOVA, followed by Fisher protected least significant differences analysis.

The SOCS were initially thought to be a feedback mechanism to dampen or terminate signaling responses to cytokines (26, 27). More recently, SOCS have been shown to mediate cross-talk between cytokines using the JAK-STAT pathway (i.e. SOCS2 expression is induced by IL-1β; expression of SOCS2 and CIS is increased only by GH). Second, forced expression of SOCS3 was just as effective as IL-1β in inhibiting the GH-dependent activation of the ALS promoter. Others have also reported that forced expression of SOCS3 inhibits the GH and prolactin activation of other STAT5-dependent promoters (44, 45). Finally, the ability of SOCS3 to mediate GH resistance in H4-II-E cells is physiologically relevant, as identical results were obtained in primary rat hepatocytes in which the GH-regulated ALS and IGF-I genes are transcribed (21, 25). Overall, they indicate that SOCS3 mediates at least a portion of the inhibitory effects of IL-1β on the GH-dependent increase of ALS gene transcription.

The SOCS were initially thought to be a feedback mechanism to dampen or terminate signaling responses to cytokines (26, 27). More recently, SOCS have been shown to mediate cross-talk between cytokines using the JAK-STAT pathway (i.e. SOCS1 mediates the inhibition of IL-4-induced gene expression by IFN-γ (Ref. 33), SOCS3 mediates the inhibition of IFN-γ and IFN-α-induced gene expression by IL-10 (Ref. 36)). Here, we extend these observations by showing that IL-1β, a cytokine that uses a completely different set of signaling molecules (40, 41), induces SOCS3 to modulate GH signaling in liver cells. This modulation of GH action may extend to other tissues as IL-1β increases SOCS3 mRNA in mouse pituitary, hypothalamus, bone marrow, and in the corticotroph A1T20 cells (28, 46).

As the other SOCS, SOCS3 features a nonconserved N-terminal region, a central SH2 domain, and the SOCS signature motif of 40 amino acids at the carboxyl end (26, 27). The roles of these domains in mediating the interference of GH signaling and the reduction in the abundance of activated STAT5 by SOCS3 has yet to be described, but clues are available from other model system. In vitro, the SH2 domain of SOCS1 binds to tyrosine phosphorylated JAK2, and allows an additional N-terminal domain of 12 residues to inhibit its catalytic activity (47). SOCS3 also binds to JAK2, but is unable to inhibit directly the catalytic activity of JAK1 or JAK2 (48, 49). Because inhibitory actions of SOCS3 on IL-6 signaling require the SH2 domain (48), SOCS3 may inhibit GH action by interfering with the binding of JAK2 and/or of STAT5 to activated receptor complexes. This mode of inhibition is used by CIS, which competes STAT5 for binding to activated IL-3 and erythropoietin receptors (37, 50). In contrast, the SOCS box is dispensable for inhibition of JAK-STAT signaling (48), and its role may be to direct SOCS containing complexes to proteosomal degradation by interacting with elongins B and C (51). Degradation of SOCS may be necessary for repeated cycles of cytokine action, particularly for regulation of hepatic genes whose transcription depends on a pulsatile pattern of GH signaling (52, 53).

Induction of SOCS3 may not be the only mechanism by which IL-1β antagonizes signaling by the GH receptor and the JAK-STAT pathway in liver cells. First, IL-1β could reduce the abundance of the GH receptor (21, 22). This appears unlikely under our experimental conditions, as abundance of the GH receptor mRNA was identical in GH-treated liver cells with or without IL-1β, and IL-1β did not decrease the GH-dependent induction of SOCS2 (Fig. 5 and results not shown). Second, IL-1β could also increase the abundance/activity of a protein that specifically sequester STAT5 in inactive complexes, similar to the recently described protein inhibitors of activated STAT1 or of activated STAT3 (54, 55). This would add to the inhibitory actions of IL-1β on STAT5-mediated transcription, but would not explain the decreased levels of activated STAT5 in cells treated with both GH and IL-1β. Finally, IL-1β could add to the effects of SOCS3 by inducing a tyrosine phosphatase acting on activated GH receptor, JAK2, or STAT5 (56).

Our results also have general significance regarding GH action and the regulation of circulating IGF-I. First, GH resistance also occurs in situations in which inflammatory cytokines are not induced such as fasting and undernutrition (57). Our data raised the possibility that factors associated with these situations can also cause GH resistance in liver and other tissues by inducing the expression of SOCS. Second, they also suggest that decreased synthesis of ALS during diseases associated with GH resistance is an additional factor contributing to lower circulating levels of IGF-I (13–15). In support of this idea, we have observed a 30% reduction in serum IGF-I in mouse with a single null ALS allele, despite unaltered hepatic synthesis of IGF-I (3). This effect reflects the need for a large excess of serum ALS relative to IGF-I for the efficient capture and retention of newly synthesized IGF-I into slowly turning over complexes of 150 kDa (5).

Acknowledgments—We thank L. Hirschberger and Dr. M. H. Stipanuk for their help in the studies with primary hepatocytes.

3 Y. R. Boisclair and G. T. Ooi, unpublished results.
