Inhibition of growth hormone action in models of inflammation

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Bergad, Pearl L., Sarah Jane Schwarzenberg, Jeffrey T. Humbert, Michelle Morrison, Sherani Amarasinghe, Howard C. Towle, and Susan A. Berry. Inhibition of growth hormone action in models of inflammation. Am J Physiol Cell Physiol 279: C1906–C1917, 2000.—Growth hormone (GH) action is attenuated during the hepatic acute-phase response (APR). To understand this attenuation, we asked whether GH and cytokine-signaling pathways intersect during an APR. In hypophysectomized rats treated with lipopolysaccharide (LPS), accumulation of activated signal transducer and transcription activator 5 (Stat5) in hepatic nuclei in response to GH and its binding to a GH response element (GHRE) from the serine protease inhibitor (Spi) 2.1 promoter are diminished in a time-dependent manner. Similarly, accumulation of activated Stat5 in hepatic nuclei in response to LPS and its binding to a high-affinity sis-inducible element (SIE) are also diminished by the simultaneous administration of GH. In functional assays with primary hepatocytes, LPS-stimulated monocyte-conditioned medium (MoCM) inhibits the GH response of Stat5-dependent Spi 2.1 reporter activity but induces Stat3-dependent Spi 2.2 reporter activity, as in an APR. Similar results are obtained when hepatocytes are treated with either tumor necrosis factor-α (TNF-α) or interleukin (IL)-1β. TNF-α, IL-1β, and IL-6 also inhibit GH-induced Spi 2.1 mRNA expression in hepatocytes. Thus inhibition of the GH signaling pathway during an APR results in reduced expression of GH-responsive genes.

INFLAMMATORY STIMULI, such as thermal burns or administration of turpentine or lipopolysaccharide (LPS), result in a series of coordinated changes in expression of a group of hepatic genes. These alterations, known collectively as the hepatic acute-phase response (APR), occur in part at the level of transcription and are important in the restoration and maintenance of homeostasis after injury (3, 15, 24, 33, 34). These events are mediated predominantly by cytokines released by monocytes and hepatic macrophages in response to these stimuli (52). These cytokines include interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) (9, 10). Although cytokines stimulate increased expression of one subgroup of hepatic genes, expression is decreased in another subgroup, referred to as negative acute-phase reactants (30, 31).

Several growth hormone (GH)-responsive genes are among the negative acute-phase reactants, suggesting that factors responding to GH may be specifically targeted during an APR. There is evidence that the suppression of GH action has clinical consequences when inflammation is severe or chronic. Suppression of linear growth is a hallmark of chronic inflammation in childhood, contributing significantly to the morbidity of inflammatory disease for children. For example, in juvenile rheumatoid arthritis, plasma insulin-like growth factor I (IGF-I) and IGF binding protein-3 levels, both of which are under direct GH regulation, are reduced (11). Patients with trauma or sepsis also have decreased IGF-I levels (18, 23, 49). Treating such patients with GH before the development of inflammation may, however, significantly reduce their ability to restore homeostasis. This is one of the proposed mechanisms for the excess mortality in critically ill patients treated with GH (58). Thus the balance between the need to promote homeostasis through the APR and the need for growth of essential tissues is a key survival issue in prolonged or chronic inflammatory illness.

To understand how the APR interferes with the transcription of GH-responsive genes, we evaluated the APR and GH responses of the rat serine protease inhibitor 2 (Spi 2) locus. Spi 2.1 is GH responsive and a positive acute-phase reactant. Its homologue, Spi 2.2, however, is not GH responsive but is a positive acute-phase reactant. During an inflammatory response after turpentine administration, the Spi 2.1 mRNA level decreases to 20% of its normal level in 24 h. Within this period the normally low Spi 2.2 mRNA level increases sevenfold (42, 50, 51). We therefore examined the divergent regulation of these two highly homologous genes in an effort to understand the interaction between inflammatory and GH effects.

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After the binding of GH to its receptor, Jak2 from the JAK family of tyrosine kinases is activated and becomes associated with the receptor (22, 29, 55, 62, 68). Signal transducers and activators of transcription (STAT) proteins 1, 3, and 5 are recruited to the receptor complex and become tyrosine phosphorylated (6, 7, 16, 20, 45, 53, 56, 60, 67). Further phosphorylation of STAT proteins at serine residues is followed by their dimerization and translocation to the nucleus. The binding of STAT dimers to interferon-γ-activated sites (GAS) on target genes results in the induction of transcription of these genes (17, 19). We delineated a GH response element (GHRE) in the Spi 2.1 promoter that contains two GAS sites recognized by Stat5, and we purified a GH-inducible nuclear factor that contains Stat5 (4, 69). In functional assays, the GHRE sequence is necessary for the GH-dependent induction of transcription of Spi 2.1 reporter fusion genes in primary rat hepatocytes (4). In COS7 cells, cotransfection of a Stat5 cDNA expression plasmid, but not a Stat1 or Stat3 cDNA expression plasmid, resulted in the GH-dependent activation of a GHRE reporter fusion gene, indicating that stimulation of gene expression via the GHRE is Stat5 specific (66).

The APR also involves similar JAK/STAT signaling pathways. Binding of IL-6, a predominant ligand in the induction of positive acute-phase reactants (14, 24) to its receptors, leads to the activation and translocation of Stat3 to the nucleus (2, 5, 45, 47, 48, 63, 64, 71). In primary hepatocyte cultures, treatment with IL-6 results in the activation of Stat3 and expression of Stat3-dependent Spi 2.2-CAT reporter activity and endogenous Spi 2.2 mRNA (5). Thus Stat3 is likely the major mediator of transcription of Spi 2.2 and many other positive APR genes.

Addition of other cytokines released during an APR, such as TNF-α and IL-1β, to hepatocyte cultures results in the induction of the GH-induced mRNA expression of IGF-I (12, 13, 35, 59, 65). We were therefore interested to find out whether they play a similar role in the downregulation of other GH-responsive genes, such as Spi 2.1, during an APR.

We hypothesized that the divergence in expression of the Spi 2 genes during an APR is due, in part, to changes in the patterns of activation of STAT proteins in response to competing stimuli. To examine this, we assessed the responses of STAT proteins to GH in whole animals during an APR to determine whether these stimuli are capable of modulating each other’s actions through STAT pathways. We also examined their interactions in primary rat hepatocytes under different treatment conditions by utilizing the responses of Spi 2.1 and Spi 2.2 reporter fusion genes and mRNA expression as respective models of Stat5 and Stat3 action.

MATERIALS AND METHODS

Cytokines. Human GH was obtained from Eli Lilly (Indianapolis, IN). Human TNF-α (210-TA), rat IL-1β (501-RL), and rat IL-6 (206-IL) were obtained from R & D Systems (Minneapolis, MN).

Treatment of animals. All animals were handled in accordance with experimental protocols approved by the University of Minnesota Institutional Committee on the Care and Use of Animals. Normal or hypophysectomized male Sprague-Dawley rats (100–125 g body wt) were obtained from the supplier (Harlan Sprague Dawley, Indianapolis, IN). Hypophysectomized rats were observed for 3 wk after arrival to confirm growth failure. Individual animals were injected with LPS (Sigma Chemical, St. Louis, MO) at a dose of 1 mg/150 g body wt and were killed at 1, 2, 3, or 4 h after injection. Nuclei were isolated from freshly excised livers and extracted for proteins according to previously published protocols (6).

A parallel series of hypophysectomized rats was similarly treated with LPS for 1, 2, 3, or 4 h, and, to determine the interactions of GH effects with the APR, each rat also received a dose of GH (30 μg/100 g body wt) 1 h before it was killed. Three different series of rats were subjected to these treatments, and hepatic nuclear extracts were prepared.

Oligonucleotides. The sequences of the Spi 2.1 oligonucleotide, GHRE, and SIE duplexes are shown in Table 1. The GHRE and SIE duplexes were extended with the Klenow fragment of Escherichia coli DNA polymerase I and radiolabeled deoxy-[γ-32P]CTP. The extended products were subsequently purified through Bio-Spin 6 columns (Bio-Rad Laboratories, Hercules, CA).

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSA), as previously described (4), were carried out with hepatic nuclear proteins extracted from treated animals and the radiolabeled GHRE or the high-affinity sis-inducible element (SIE). Briefly, 5 μg of hepatic nuclear extracts were incubated with 20 fmol of the radiolabeled probe of interest in a buffer containing 20 mM HEPES (pH 7.6), 10% glycerol, 2 mM MgCl2, 5 mM CaCl2, 0.1 mM spermidine, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg poly dI.dC. Final KCl concentration was adjusted to 50 mM. After 30 min of incubation at 30°C, the binding mixture was loaded onto a nondenaturing, 5% polyacrylamide gel. After 2 h of electrophoresis at 150 V, the gel was dried and exposed to film. Quantitation of the shifted complexes on the film was performed using the Kodak Digital Science ID Image Analysis Software.

Antibodies. All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Stat5b antibody (sc-835) is a polyclonal antibody generated against amino acids 711–727 of the Stat5b protein.
at the carboxy terminus of mouse Stat5b. This antibody reacts with both Stat5a and Stat5b. Antibody to Stat3 (sc-7179) is a polyclonal antibody generated against amino acids 50–240 at the amino terminus of human Stat3. Antibody to phosphotyrosine (sc-7020) is a mouse monoclonal antibody specific for detection of phosphotyrosine-containing proteins.

Immunoprecipitations and immunoblots. Immunoprecipitations were performed, as previously reported (25), with hepatic nuclear extracts from treated animals and an antibody to phosphotyrosine. Briefly, aliquots containing 100 μg of nuclear proteins were incubated with 2 μg of phosphotyrosine antibody in RIPA buffer (50 mM Tris·HCl, pH 7.5, 1% Nonidet P-40, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, and 0.1% sodium deoxycholate) containing, in addition, 0.2 mM PMSF, 1 mg/ml aprotinin, and 10 mM NaF. After 1 h of mixing, protein A agarose (120 μg) was added to each mixture, and end-to-end mixing was continued for 16 h at 4°C. The mixtures were centrifuged at 700 g for 5 min at 4°C. The resulting pellets were washed and solubilized in SDS electrophoresis sample buffer, and aliquots were loaded onto a 7.5% SDS-PAGE gel. Immunoblotting with antibodies to either Stat3 or Stat5 was performed as reported previously (4, 6). Positive signals were detected with the enhanced chemiluminescence detection system (Amersham Life Science, Arlington Heights, IL) and quantitated using Kodak Digital Science ID Image Analysis Software.

Plasmid construction. The constructions of Spi 2.1 (−275/+85) and Spi 2.2 (−319/+85) into the Hind III/Pst I sites of the parent chloramphenicol acetyltransferase (CAT) reporter plasmid pCAT(An), and Spi 2.1 (−147/−102)−TKCAT, containing four copies of the GHRE, were described previously (5,69).

Monocyte-conditioned medium. Monocyte-conditioned medium (MoCM) was prepared according to the method of Darlington et al. (10). In brief, 20 ml of human blood were centrifuged at 400 g for 10 min at room temperature. The cell pellet, enriched for leukocytes, was resuspended in RPMI medium (GIBCO BRL, Rockville, MD) and layered over 5 ml of Ficoll-Hypaque. Polymorphonuclear leukocytes and granulocytes were sedimented by centrifugation at 400 g for 30 min. Cells located at the interface of the supernatant and the Ficoll-Hypaque were isolated by suction, washed twice with PBS, and plated in RPMI medium plus 10% fetal bovine serum. Monocyte cultures were stimulated to increase production of AP-activating agents by the addition of 10 μg/ml of LPS for 48 h. The MoCM was then collected and frozen in aliquots at −20°C.

Functional assays. Primary hepatocytes were isolated using the collagenase method, according to previously published protocols (4). After a 4-h attachment period, cells were transfected with either Spi 2.1-CAT or Spi 2.2-CAT using Lipofectin reagent (Life Technologies, Grand Island, NY) in modified Williams E medium with 27.5 mM glucose for 12–14 h. After removal of the Lipofectin and subsequent washing, Matrigel (Life Technologies, Grand Island, NY) at 667 μg/35-mm culture dish was added to the medium, and the hepatocytes were cultured in the presence or absence of 50 ng/ml GH. For experiments involving specific cytokines, 1 mg/ml of TNF-α, 1 ng/ml of IL-6, or 0.5 ng/ml of IL-1β was added in addition to GH. At the end of 24 h, all media were replaced, and fresh GH and/or cytokines were added. At the end of another 24 h, the cells were harvested and lysed in Reporter Lysis Buffer (Promega, Madison, WI) for CAT assays. Results of the assays are expressed as percent conversion of chloramphenicol to its acetylated forms as determined by phosphor screen autoradiography (Molecular Dynamics, Sunnyvale, CA). Each experiment was repeated three times with freshly isolated hepatocytes. At least two different plasmid preparations of each reporter fusion gene were tested in these experiments.

For assays with MoCM, isolated hepatocytes were transfected with either Spi 2.1-CAT, Spi 2.2-CAT, or Spi 2.1 (−147/−102)−TKCAT as described above, and then stimulated with either no hormones, GH, IL-6, or GH + MoCM. For hepatocytes to be treated with GH + MoCM, one-half of the medium from each plate was replaced with MoCM (i.e., MoCM comprised 50% of total medium). This concentration was chosen on the basis of work by previous investigators (10) and our own dose-response studies. For hepatocytes not to be treated with MoCM, one-half of the medium from each plate was replaced with RPMI medium. GH or IL-6 was then added to the appropriate plates. This protocol was repeated 24 h later. After another 24 h, cells were harvested for CAT assays as described above.

Northern blots. For hepatocyte mRNA studies, Matrigel was added to the modified Williams E medium with 27.5 mM glucose after the attachment period, and the cells were allowed to rest for 16 h. GH and/or specific cytokines were then added. After 24 h of culture, the hepatocytes were harvested and RNA was extracted with TRIzol reagent (GIBCO BRL). Northern blots prepared with these RNAs were first probed with a Spi 2.1 oligonucleotide complementary to its reactive region (51) that had been 5′-labeled with T4 polynucleotide kinase and [γ-32P]ATP. The blots were then probed simultaneously with cDNAs of Spi 2.2 from its unique 3′-untranslated region and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). These cDNAs were radiolabeled using the Oligo-labelling Kit (Amersham Pharmacia Biotech, Piscataway, NJ) and deoxy-(α-32P)CTP. All quantitations of Spi 2.1 or Spi 2.2 mRNAs were normalized to those of GAPDH mRNA, as determined by phosphor screen autoradiography.

RESULTS

LPS treatment of hypophysectomized rats reduces Stat5 binding to the GHRE during a GH response. To study the potential interactions between LPS and GH treatments in whole animals, we examined the effects of treatment with LPS (1 mg/150 g body wt) in two groups of hypophysectomized rats. One group was given LPS alone and killed at 1, 2, 3, or 4 h after treatment; the second group received, in addition, GH (30 μg/100 g body wt) 1 h before death. The results of EMSA that use hepatic nuclear proteins extracted from these animals and radiolabeled GHRE are shown in Fig. 1. We have shown previously (4, 60) that GHRE binds specifically to activated Stat5 and that administration of GH to an untreated hypophysectomized rat 1 h before it is killed results in significant induction of Stat5 binding (lane 2 vs. lane 1). LPS treatment preceding GH administration diminished this binding in a time-dependent manner, with only 10% of the expected GH-induced Stat5 binding present 4 h after LPS treatment (lanes 3–6). A parallel series of rats treated with LPS alone did not show any Stat5 binding to the GHRE (lanes 7–10). Therefore, events occurring during the progression of an AP result in a reduction of GH-induced Stat5 binding. GH treatment reduces Stat3 binding to the SIE during the early stage of LPS treatment of hypophysectomized rats. To examine the effects of GH administra-
tion on changes in Stat3 binding during the progression of an APR, we carried out EMSA of the same hepatic nuclear extracts using radiolabeled SIE. Figure 2 shows that, as previously reported (4), GH treatment activated Stat1 and Stat3, resulting in the formation of three complexes containing Stat3 homodimers, Stat1 homodimers, and Stats 1 and 3 heterodimers (lane 2 vs. lane 1). LPS treatment alone led to maximal formation of a Stat3 complex 1 h after treatment (lane 7). This binding decreased over the next 3 h to 56% at 4 h after LPS treatment (lanes 8–10). GH treatment, given at the same time as LPS treatment, diminished the binding of Stat3 stimulated by LPS within the 1st h by 36% (lane 3 vs. lane 7). However, GH treatment given at later time points in LPS treatment did not result in any notable differences in Stat3 binding (lanes 4–6) compared with those treated with LPS alone (lanes 6–10).

In contrast to Stat3, the time course of LPS-stimulated binding of Stat1 is slower. Binding to Stat1 was not evident until 2 h after treatment. It increased 1.5-fold during the next hour and remained constant 1 h later (lanes 7–10). Its activation by GH alone, evident 1 h after treatment (lane 2), remained essentially unchanged during LPS treatment (lanes 3–6). GH administration during LPS treatment did not alter the total amount of Stat1 binding observed (lanes 5 and 6 vs. lanes 9 and 10). Thus early GH administration reduces LPS-stimulated Stat3 binding but does not affect Stat1 binding.

Simultaneous treatment with LPS and GH reduces the accumulation of tyrosine-phosphorylated Stats 3 and 5 in the nucleus. The decreases in Stat5 binding to the GHRE and Stat3 to the SIE in extracts from hypophysectomized rats treated with both LPS and GH could be due to an inhibition of DNA binding or decreases in the amounts of these STAT proteins in the nuclei. To delineate between these two possibilities, we performed immunoprecipitations of these extracts with an antibody to phosphotyrosine, and we probed blots of the resultant immunoprecipitates with an antibody either to Stat5 (see Fig. 3) or to Stat3 (see Fig. 4).

Figure 3 shows that there was a time-dependent decrease in the amount of tyrosine-phosphorylated Stat5 in the nuclei of rats treated with LPS and GH (Fig. 3A). LPS treatment thus results in a decrease in GH-activated accumulation of tyrosine-phosphorylated Stat5 in the nucleus. LPS treatment by itself also resulted in some accumulation of tyrosine-phosphorylated Stat5 in the nuclei (Fig. 3B) compared with an untreated animal, although no LPS-induced binding of Stat5 to the GHRE probe was observed (Fig. 1, lanes 7–10). It is possible that, in addition to causing a decrease in accumulation of GH-activated Stat5 in the nucleus, LPS treatment also stimulates an inhibition of DNA binding.

Figure 4 shows that LPS treatment of hypophysectomized rats caused an accumulation of tyrosine-phosphorylated Stat3 in the nuclei 1 h after treatment. This amount decreased over the next 3 h (Fig. 4B). When GH was administered simultaneously with LPS, there was a marked decrease in the amount of tyrosine-phosphorylated Stat3 in the nuclei within the 1st h (Fig. 4A, H+LPS+GH, 1 h) compared with LPS treat-
ment alone (Fig. 4B, H+LPS, 1 h). GH treatment (given always 1 h before the animal was killed) at later time points after LPS treatment resulted in only slight decreases in the amounts of tyrosine-phosphorylated Stat3 in the nucleus (Fig. 4A, H+LPS+GH, 2–4 h) compared with LPS treatment alone. Thus GH administration reduces the accumulation of tyrosine-phosphorylated Stat3 in the nucleus in the early phase after LPS treatment, consistent with the results observed in SIE binding.

**MoCM inhibits the GH response of Spi 2.1-CAT.** Our studies in whole animals to this point indicated that the GH-activated and LPS-stimulated STAT pathways interfere with each other and may account, in part, for the reduction in expression of some negative acute-phase reactants during an APR. To better understand this aspect of the complex events that are taking place during an APR and to further dissect the roles of individual cytokines in influencing STAT activation, we concentrated our further investigations on primary hepatocyte culture. The GHRE in the Spi 2.1 promoter has served as an in vitro model system for Stat5 activation (66). Similarly, a Spi 2.2 promoter reporter fusion gene has served as an in vitro model system for Stat3 activation (5, 32). We therefore conducted functional assays utilizing Spi 2.1 and Spi 2.2 promoter reporter fusion genes and studied their responses to different stimuli to dissect aspects of the hormonal milieu generated during an APR.

MoCM has been used as a means for initiating an APR in human hepatoma cells (10). We therefore tested the effects of MoCM on the GH response of Spi 2.1-CAT in hepatocytes (Fig. 5A). In the absence of MoCM, Spi 2.1-CAT exhibited a robust GH response (GH vs. UT), but in its presence, the activity of Spi 2.1-CAT in response to GH was almost completely inhibited. This inhibitory effect was also observed with Spi 2.1 (−147/−102)TKCAT, a reporter fusion gene containing only four copies of the GHRE linked to the TK promoter (Fig. 5B). Thus the inhibition of the GH response of Spi 2.1 occurs via the GHRE and does not require other regions of the Spi 2.1 promoter. The GHRE contains two Stat5 binding sites (4), and its response to various stimuli is Stat5 specific (66). Thus MoCM-mediated reduction in promoter activity is consistent with the LPS-mediated inhibition of GH-stimulated Stat5 binding to the GHRE shown earlier. In contrast, Spi 2.2-CAT, which is not GH responsive but responds to IL-6 via the Stat3 pathway (5), was activated in the presence of the MoCM (Fig. 5C). Taken together, these data indicate that MoCM contains cytokines that are capable of activating Spi 2.2-CAT and, at the same time, inhibiting the GH response of Spi 2.1-CAT.

**Both TNF-α and IL-1β inhibit the GH response of Spi 2.1-CAT.** By means of radioimmunoassays, we found that MoCM contains TNF-α (487 pg/ml), IL-1β (140 pg/ml), and IL-6 (490 pg/ml), all cytokines known to be active during an APR (3, 10). We have previously found...
that IL-6, at 5 ng/ml, will induce Spi 2.1-CAT activity in hepatocyte culture, and that addition of IL-6 to GH does not alter the GH response of Spi 2.1-CAT (5). However, at the lower concentration of 1 ng/ml, similar to that found in MoCM, IL-6 addition resulted in only a modest activation of Spi 2.1-CAT. Simultaneous addition of TNF-α, IL-1β, and IL-6 led to a reduction of this response (Fig. 6).

We next questioned whether TNF-α or IL-1β might be the agent in the MoCM that reduced the response of the Spi 2.1 promoter to GH. To examine this, we added TNF-α or IL-1β to cultured hepatocytes and assessed the GH response of Spi 2.1-CAT in their presence (Fig. 6). Addition of TNF-α at 1 ng/ml reduced activity of Spi 2.1-CAT in response to GH by 70%. Addition of IL-1β at 0.5 ng/ml led to a similar (62%) reduction of Spi 2.1-CAT activity. Simultaneous addition of IL-6, IL-1β, and TNF-α reduced Spi 2.1-CAT activity by 85%. Neither TNF-α nor IL-1β by itself induced any Spi 2.1-CAT activity. Therefore IL-1β and TNF-α, singly or in combination, reduce the response of the Spi 2.1 promoter to GH.

To determine whether TNF-α or IL-1β had any effect on the IL-6 induction of the Spi 2.2 promoter, we carried out similar functional assays using Spi 2.2-CAT reporter fusion genes (Fig. 7). Treatment with IL-6 alone resulted in a substantial induction of Spi 2.2-CAT activity, whereas addition of TNF-α, IL-1β, or GH, singly or together, resulted in minimal induction of Spi 2.2-CAT activity. Simultaneous addition of IL-6, IL-1β, and TNF-α, with or without GH, resulted in modest decreases of the IL-6 response of Spi 2.2-CAT. Thus the inhibitory effects of TNF-α and IL-1β on IL-6 action are less than those on Spi 2.1. Their overall effects are similar to those exhibited by the MoCM.

**TNF-α, IL-1β, and IL-6 inhibit Spi 2.1 mRNA induction by GH in isolated primary hepatocytes.** To determine whether the in vitro effects of TNF-α, IL-1β, and IL-6 on the GH response of Spi 2.1-CAT were due to direct effects on Spi 2.1 promoter activity, we carried out similar functional assays using Spi 2.1-CAT reporter fusion genes (Fig. 5). Treatment with IL-6 alone resulted in a substantial induction of Spi 2.1-CAT activity, whereas addition of TNF-α, IL-1β, or GH, singly or together, resulted in minimal induction of Spi 2.1-CAT activity. Simultaneous addition of IL-6, IL-1β, and TNF-α, with or without GH, resulted in modest decreases of the IL-6 response of Spi 2.1-CAT. Thus the inhibitory effects of TNF-α and IL-1β on IL-6 action are less than those on Spi 2.1. Their overall effects are similar to those exhibited by the MoCM.
IL-6 on the response of the Spi 2.1 promoter to GH correlate with their effects on endogenous Spi 2.1 mRNA expression, we cultured primary hepatocytes in the presence of GH with or without these cytokines for 24 h. Figure 8, top, shows a representative Northern blot of RNA extracted from these cells and probed with a radiolabeled Spi 2.1 oligonucleotide. Figure 8, bottom, shows the mean values obtained from four similar blots. GH treatment typically resulted in an 8- to 10-fold induction (on average) of Spi 2.1 mRNA, expressed as 100% in Fig. 8, bottom. Addition of either TNF-α (0.5 ng/ml) or IL-1β (0.25 ng/ml) reduced this induction by almost 60%. Treatment with IL-6, which is capable of inducing Spi 2.1 promoter activity (5), did not result in induction of endogenous Spi 2.1 mRNA. At 0.5 ng/ml, IL-6 treatment actually reduced GH-induced Spi 2.1 mRNA expression by 65%. Simultaneous addition of TNF-α, IL-1β, and IL-6 completely blocked the GH-stimulated induction of Spi 2.1 mRNA. Thus TNF-α, IL-1β, and IL-6, given singly or in combination, inhibit Spi 2.1 mRNA induction by GH.

Reprobing of the Northern blot shown in Fig. 8, top, with a Spi 2.2 cDNA (Fig. 9, top) demonstrates IL-6 induction of Spi 2.2 mRNA of ~10-fold. Addition of GH did not alter this induction. Addition of TNF-α or IL-1β resulted in a modest reduction of 15% (Fig. 9B). GH treatment alone did not result in any induction of Spi 2.2 mRNA. Thus the endogenous Spi 2.2 mRNA is altered in a similar manner to that of its promoter reporter fusion gene in response to GH and cytokines.

**DISCUSSION**

We examined the interactions between GH and LPS stimulation in hypophysectomized rats during the first several hours of an APR. LPS is frequently employed in inducing an APR in an animal. Although its treatment might elicit changes in cytokine levels that are different from more authentic immune responses resulting from chronic inflammations, the information we learn from the use of LPS as a model is still valuable and can guide us in further efforts to understand the interactions between GH signaling and inflammation. Because the effects on GH signaling 1 h after treatment are well documented (60), we administered GH to these LPS-treated animals 1 h before they were killed. We found time-dependent changes in the amounts of GH-responsive phosphorylated Stat5 in the liver nuclei that were caused by events occurring during an APR. These changes were not correlated to changes in the total amount of Stat5 under these conditions, because immunoprecipitations of whole cell extracts from livers of these animals with an antibody that recognises both Stat5a and Stat5b (sc-835) indicated that there was little change in the expression level of Stat5 in the liver during the first 4 h after LPS administration (data not shown). However, immunoprecipitations of nuclear extracts with an antibody to phosphotyrosine showed that the amount of GH-responsive phosphorylated Stat5 in the nuclei decreased progressively during the same time period. This decrease, down to ~40% at the end of 4 h, was somewhat less than the decrease in Stat5 binding to the GHRE observed in EMSA, indicating that there may be specific inhibition of Stat5.
binding to phosphorylated tyrosine residues on specific JAK tyrosine kinase activities to competition for an APR. The resulting mechanism involves the induction of SOCS genes by cytokines released in response to LPS, and subsequent SOCS inhibition of GH signaling (38). In this report we have shown similar data on the LPS-dependent decrease of GH-responsive Stat5 phosphorylation and DNA binding in the liver of hypophysectomized rats. Our data suggest that the hypothesis that SOCS-induced inhibition alone may account for the downregulation of GH signaling may be an oversimplification. Hepatocyte studies in our laboratory indicate that treatment with either TNF-α or IL-1β alone, under conditions that led to a reduced GH response of Spi 2.1-CAT, did not result in an increase in expression of either SOCS-2 or SOCS-3 mRNAs in the first 8 h of culture, indicating that other mechanisms of inhibition are likely at work (data not shown).

A comparison of the time courses of induction of CIS, SOCS-2, and SOCS-3 by seemingly antagonistic stimuli, such as GH (1, 38, 57), indicates that they are remarkably similar. This raises the interesting question of how a gene that is involved in the GH negative feedback loop in the normal state can become a more enduring inhibitor when it is activated during an APR with no change in the kinetics of its induction or the persistence of its mRNA. Whether there is a change in the level or the half-life of its protein product under these different conditions remains to be established. If we assume that there are changes at the protein levels, how then is the GH-signaling pathway selectively inhibited whereas that of IL-6, necessary in an APR, is not? Part of the answer may lie in the discovery of an inhibitor specific for a particular target or step in the GH-signaling pathway. To date, no CIS or SOCS protein has been shown to provide this degree of specificity. GH activates Stat1 and Stat3, in addition to Stat5, in the liver of hypophysectomized rats, whereas LPS treatment results only in the activation of Stat1 and Stat3. This suggests that there is an inhibitory step during the induction of the APR that specifically targets the activation of Stat5 by GH. The results we report here and those recently published (38) support this view.

Another possible mechanism for the interference of the GH-signaling pathway could involve the tyrosine phosphatases SHP-1 and SHP-2. During a GH response, SHP-1 translocates to the nucleus and associates with phosphorylated Stat5b, suggesting that it can participate in the dephosphorylation of nuclear Stat5b (46). At the same time, SHP-1 is also associated with Jak2 and appears to be involved in the attenuation of Stat5b activation (46). At the same time, SHP-1 is also associated with Jak2 and appears to be involved in the attenuation of Stat5b activation. The transient expression is activated with Jak2 and appears to be involved in the attenuation of Stat5b activation. The transient expression is activated with Jak2 and appears to be involved in the attenuation of Stat5b activation (46). At the same time, SHP-1 is also associated with Jak2 and appears to be involved in the attenuation of Stat5b activation. The transient expression is activated with Jak2 and appears to be involved in the attenuation of Stat5b activation. The transient expression is activated with Jak2 and appears to be involved in the attenuation of Stat5b activation (46). At the same time, SHP-1 is also associated with Jak2 and appears to be involved in the attenuation of Stat5b activation.
tion of GH-activated JAK activity (21). On the other hand, SHP-2 is a negative regulator in the IL-6 induction of positive acute-phase reactants and acts by downregulating JAK activity (28). The interactions of these phosphatases during an APR thus may also be involved in the mutual modulation of GH- and LPS-stimulated cytokine signaling.

In hypophysectomized rats treated with LPS alone, we observed maximal binding of Stat3 at 1 h after LPS administration. Interestingly, this initial activation can be modulated by simultaneous administration of GH and may in part account for the GH attenuation of an APR due to thermal injury (26, 27, 44). This modulation may be due, in part, to the simultaneous activation of Stat1 by GH that may sequester some Stat3 into the formation of Stat1/Stat3 heterodimers. It is not clear from current literature what role, if any, this heterodimer plays in vivo. GH administration at 2 h or later after LPS treatment did not alter the amount of activated Stat3 in liver nuclei, indicating that signaling events involving the activation of Stat3 take place within the first 2 h after LPS administration.

GH activation of Stat3 in the hypophysectomized rat does not result in the transcription of Spi 2.2, a positive acute-phase reactant. The explanation for this apparent discrepancy is not understood. The amount of Stat3 activated by GH was less than when the animal was treated with LPS alone and might contribute to this observation. The simultaneous activation of Stats 1 and 5 by GH may affect both the amount of Stat3 activated and its eventual binding to the promoters of target genes. Interactions among different STAT proteins or competition for binding sites may determine the transcriptional outcome for a responsive gene. For example, Stat5b modulates the Stat1-mediated transcription of interferon regulatory factor-1 in response to prolactin by protein-protein interactions that do not involve DNA binding (37). On the other hand, FcγR1, an interferon γ-responsive gene, contains a GAS region that binds both Stat1 (43) and Stat5 (4), resulting in the formation of complexes containing both dimers and tetramers of these respective factors. However, binding of its GAS region to Stat1 alone is sufficient to initiate transcription (43).

Utilizing Spi 2.1 and Spi 2.2 promoter reporter fusion genes, we investigated their responses to different stimuli as indicators of functional Stat5 and Stat3, respectively. MoCM, produced by isolated human monocytes stimulated with LPS, was able to attenuate the GH response of Spi 2.1-CAT and at the same time induce Spi 2.2-CAT activity. The divergent responses of these Spi promoter reporter fusion genes to MoCM are similar to those observed for their respective mRNAs during an APR in an intact rat (51). Thus factors present in MoCM are capable of reproducing an in vivo observation during an APR. An analysis of MoCM showed that, similar to previous reports (10), it contains substantial amounts of IL-6, TNF-α, and IL-1β. The reduction of Spi 2.1-CAT activity in hepatocytes cultured in the presence of GH and either exogenous TNF-α or IL-1β demonstrated that both TNF-α and IL-1β are capable of inhibiting the GH response of Spi 2.1-CAT. Both TNF-α and IL-1β, at 10 ng/ml, have also been shown to inhibit GH-responsive expression of IGF-1 and Spi 2.1 mRNAs (54, 59) in hepatocyte cultures. We now show that, in the presence of even low concentrations of these two cytokines, at 0.25 or 0.5 ng/ml, both GH-responsive Spi 2.1 promoter activity and its endogenous mRNA expression are markedly inhibited.

Spi 2.2 belongs to type II positive acute-phase reactants that require IL-6, but not IL-1, in their induction; in contrast, type I reactants require both IL-6 and IL-1 (32). Treatment of hepatocytes with either TNF-α or IL-1β does not result in induction of Spi 2.2-CAT activity. Moreover, their combined addition results in an inhibition of IL-6-induced Spi 2.2-CAT activity. The inhibitory effects of these two cytokines on the GH-induced Spi 2.1-CAT activity are more prominent and are also noted in expression of their endogenous mRNA. Thus their role in the regulation of the Spi genes during an APR appears to be primarily inhibitory.

LPS administration to hypophysectomized rats leads to the accumulation of a small amount of phosphorylated Stat5 in the hepatic nucleus. Treatment of hepatocytes with IL-6, an important ligand released during an APR, results in some, albeit low, Spi 2.1-CAT activity. Preliminary data from our laboratory on GHRE mutation studies in hepatocytes indicate that IL-6 induction of Spi 2.1-CAT activity occurs via the GHRE, a sequence that is Stat5 specific (66). Moreover, treatment of rats with complete Freund’s adjuvant, another APR stimulant, has resulted in the stimulation of Stat5, in addition to Stat3 (48). Thus, in addition to activating Stat3 (2, 5), IL-6 appears to be capable of activating Stat5 to some extent, resulting in Stat5-specific (GHRE) promoter activity. The extent of this activation, however, does not give rise to the production of endogenous Spi 2.1 mRNA. IL-6 treatment of hepatocytes actually attenuates the accumulation of Spi 2.1 mRNA in response to GH. These conflicting effects of IL-6 on Spi 2.1 expression may indicate post-transcriptional regulation. The attenuation of Spi 2.1 mRNA induction by IL-6 in hepatocytes correlates well with the observation that, in the intact animal, turpentine treatment results in a decreased induction of Spi 2.1 mRNA (51).

In the dynamic situation within a cell, interactions among different factors and their modulations of each other’s functions may determine the eventual transcriptional outcomes of target genes. Signaling by competing ligands results in complex events that can be mutually regulated at multiple steps. Using the hepatocyte model system, we have shown that IL-6, IL-1β, and TNF-α act in concert to reduce the GH-induced expression of Spi 2.1, a Stat5-mediated gene. We propose that, during an APR, inhibition specific to the GH/Stat5 signaling pathway leads to the downregulation of Stat5-mediated GH-responsive genes such as Spi 2.1.
INHIBITION OF GH ACTION IN INFLAMMATION

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