Opposing effects of pituitary leukemia inhibitory factor and SOCS-3 on the ACTH axis response to inflammation

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Chesnokova, Vera, Anastasia Kariagina, and Shlomo Melmed. Opposing effects of pituitary leukemia inhibitory factor and SOCS-3 on the ACTH axis response to inflammation. Am J Physiol Endocrinol Metab 282: E1110–E1118, 2002.—We have shown that leukemia inhibitory factor (LIF) and suppressor of cytokine signaling (SOCS)-3 are expressed in the hypothalamus and pituitary and that LIF induces proopiomelanocortin (POMC) and ACTH, whereas SOCS-3 abrogates corticotroph POMC gene transcription and ACTH secretion. Here, we determined the role of pituitary LIF and SOCS-3 in regulating hypothalamo-pituitary-adrenal (HPA) axis inflammatory responses. Murine pituitary LIF expression was induced up to eightfold after intraperitoneal injection of lipopolysaccharide or tumor necrosis factor-α, concordant with elevated plasma levels of ACTH and corticosterone. In LIF knockout (LIFKO) mice, induction of both ACTH and corticosterone were attenuated. LIF deletion was associated with elevated (P < 0.05) levels of pituitary TNF-α, interleukin (IL)-1β, and IL-6 mRNA and cytokine-inducible pituitary SOCS-3 expression. Abrogation of the HPA axis stress response and higher pituitary levels of proinflammatory cytokines observed in LIFKO mice resulted in a stronger inflammatory process, as evidenced by elevated erythrocyte sedimentation rate and increased serum amyloid A levels (P < 0.05). The results indicate that, although LIF induces ACTH, SOCS-3 acts to counterregulate the HPA axis response to inflammation.

Cytokine regulators of immune and inflammatory processes play important roles in activating hypothalamo-pituitary-adrenal (HPA) axis responses to immunological challenges. Cytokines are locally produced at the site of inflammation, and circulating plasma cytokine levels are elevated as a result of systemic inflammation. Lipopolysaccharide (LPS) administration or inflammation also induces hypothalamic and pituitary cytokines, which centrally activate the HPA axis inflammatory stress response (6, 21, 28, 29, 37).

Leukemia inhibitory factor (LIF), a member of the interleukin (IL)-6 cytokine family, is a pleiotropic cytokine with diverse biological activity (26). LIF is increased in a variety of inflammatory conditions, including rheumatoid arthritis and septic shock (40, 41). Prior LIF administration protects against lethality during endotoxemia in mice in a dose- and time-dependent manner (40). LIF also plays an important role in the development and functioning of the HPA axis (42). LIF and LIF receptor are constitutively expressed in human pituitary cells (30) and in murine hypothalamus and pituitary (39). LIF regulates differentiation and development of murine pituitary corticotrophs early in ontogenesis (29), potently induces proopiomelanocortin (POMC) gene transcription and ACTH secretion (2, 30), and potentiates corticotropin-releasing hormone (CRH) induction of POMC gene expression (11, 13). In vivo, LIF along with CRH maintains POMC expression and ACTH secretion in response to emotional stress (14).

Corticotroph cell-signaling pathways for the IL-6 cytokine family involve heterodimerization between cytokine receptor and gp130 receptor subunits with subsequent Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway activation (10, 30) and induction of cytokine-inducible suppressor of cytokine signaling (SOCS)-3 (5, 35). We demonstrated in vivo pituitary SOCS-3 induction by proinflammatory cytokines (5). In AtT20 corticotroph cells, SOCS-3 overexpression inhibits cytokine-stimulated gp130 and STAT3 phosphorylation, ACTH secretion, POMC mRNA expression, and POMC promoter activity (5, 10, 12). SOCS-3 thus behaves as an intracellular negative feedback mediator of the cytokine-endocrine interface.

Several lines of evidence support the role for hypothalamic-pituitary LIF in the chronic inflammatory process. Earlier, we demonstrated that LIF was induced almost exclusively in the hypothalamus in response to chronic systemic inflammation stimulated by mycobacterial adjuvant and to chronic local inflammation produced by intramuscular turpentine administration. In both inflammatory models, LIF-deficient mice exhibit attenuated POMC, ACTH, and corticosterone responses to inflammatory stimuli (14, 15).
present study, we examined more closely mechanisms implicating central LIF in the neuroendocrine interface in acute, rapidly evolved, LPS-induced inflammation. The results show that pituitary LIF is strongly activated in response to LPS and tumor necrosis factor (TNF)-α. LIF knockout (LIFKO) mice demonstrate lower corticosterone responses, thereby promoting the inflammatory process. Pituitary cytokine overexpression enhances cytokine-inducible pituitary SOCS-3, thus further suppressing inflammatory ACTH responses in LIFKO mice. This study shows that pituitary LIF and SOCS-3 are important for rapid regulation of the ACTH axis during acute inflammation.

METHODS

Animals. Mice heterozygous for the disrupted LIF gene (LIFKO) were kindly provided by Dr. Colin L. Stewart (Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ). Because LIFKO females exhibit defective blastocyst implantation, homozygous LIFKO animals were bred by heterozygous or homozygous male and heterozygous female mating on a B6D2F1 genetic background. After PCR DNA analysis, tail tissue, homozygous mice were sex and age matched with wild-type (WT) litters. Animals were kept in a 0600–1800 daytime cycle with free access to food and water and housed five per cage. Female mice, 8–14 wk of age were used for the experiments: LIF−/−, or WT normal, and LIF−/−, or LIFKO, mice. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

In vitro treatment. Pituitary corticotroph AtT20/D16v-F2 cells (American Tissue Culture Collection) were grown as described (15) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml streptomycin, and 100 U/ml penicillin. Cells were pretreated with 5 μM murine SOCS-3 antisense (AS) or mismatch (MM) oligonucleotides (Molecular Research Laboratories, LLC, Herndon, VA) for 36 h. For the last 16 h, cells were incubated in serum-free DMEM. Thereafter, fresh serum-free DMEM, AS or MM with or without 25 nM murine IL-6 (R&D Systems, Minneapolis, MN), were added, and after 1 h, cells were harvested for RNA isolation.

RNA was analyzed for SOCS-3 by Northern blot analysis, and each membrane was washed and reprobed for POMC mRNA. Relative abundance of SOCS-3 mRNA was determined by densitometry and normalized to levels of POMC mRNA in the same sample: the POMC-to-SOCS-3 ratio was calculated, standardized in relation to the control values taken as 1, and presented as fold increase.

In vivo treatment. Fifty micrograms of LPS (from Escherichia coli, sterile type 0111:B4, Sigma, St. Louis, MO) in 200 μl of normal saline were injected intraperitoneally into WT and LIFKO mice. Animals were killed 0.5, 1, 4, or 8 h after injection. For serum amyloid A (SAA) measurement, animals were injected with 80 μg of LPS and killed at 8 or 30 h after injection.

Murine TNF-α (0.5 μg; R&D Systems) in 200 μl of PBS was intraperitoneally injected into WT and LIFKO mice. Animals were killed 1 h after injection.

Blood collection and hormone assay. Whole blood was obtained immediately after decapitation, and plasma was collected in ice-chilled tubes containing 0.1% EDTA, separated, and stored at −70°C until assayed. Plasma ACTH (Nichols Institute Diagnostics, San Juan Capistrano, CA) and corticosterone (ICN Biomedicals, Costa Mesa, CA) were measured by commercially available RIAs. Sensitivity of ACTH and corticosterone assays was 10 pg/ml and 25 ng/ml, respectively. Inter- and intra-assay variability for ACTH was 7.3 and 3.1%, respectively; inter- and intra-assay of variability for corticosterone was 4.4 and 6.5%, respectively.

Erythrocyte sedimentation rate. Whole trunk blood was anticoagulated with EDTA and diluted 1:1 with sodium citrate. Erythrocyte sedimentation rate (ESR) was measured using Wintrobe 3 × 115-mm tubes (Becton-Dickinson, Franklin Lakes, NJ) filled up to 500 mm. Blood was obtained from untreated mice or animals killed 8 h after LPS injection, and measurements were made 16 h later.

SAA. Trunk blood was collected from untreated and LPS-treated animals, serum aliquoted, and stored at −20°C and SAA measured in 20 μl of serum by commercial ELISA SAA assay kit (Hemagen, Waltham, MA) according to the protocol.

Tissue dissection and RNA isolation. Mice were decapitated and pituitary dissected, and tissue was immediately frozen on dry ice and kept at −70°C until RNA extraction. Total tissue RNA was extracted with TRIzol reagent (Pierce, IL, Gaithersburg, MD). All experiments were performed according to the manufacturer’s instructions, in which 5–100 mg of frozen tissue were immersed in 1 ml of TRIzol solution and homogenized with a Polytron homogenizer, dissolved in diethyl pyrocarbonate water, and RNA concentration was spectrophotometrically quantitated and quality checked by gel electrophoresis.

Northern blot analysis. Northern analysis was performed using 2–25 μg of total RNA per lane. Samples were electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (Nytran 228, Schleicher & Schuell, Keene, NH), membranes were ultraviolet cross-linked, and blots were prehybridized in 1 M NaPO4, 20% SDS, and 0.1% BSA for 1 h at 65°C. 32P-labeled specific probes (106 cpm/ml) were added and membranes hybridized overnight at 65°C. Membranes were then washed in 2× standard sodium citrate (SSC) and 0.1% SDS for 30 min, 1× SSC and 0.05% SDS for 30 min, 0.5× SSC and 0.025% SDS for 1 h, and 0.1× SSC and 0.005% SDS for 1 h at 65°C and exposed to Kodak Biomax film for 2–4 h for POMC and 18S, 24–48 h for SOCS-3, and 48–72 h for LIF mRNA at −70°C.

Experiments were performed three to four times, and autoradiographs depict Northern blots from representative experiments. Seven to eight hypothalami or seven to eight pituitaries were pooled per sample in each experiment. Our experience that β-actin changes with hormonal stimulation led us to use 18S rRNA as a control housekeeping gene in all experiments. Relative abundance of LIF, POMC, and SOCS-3 mRNA was quantified with a Seikosha U1500II densitometer (Alpha Innotech) and normalized with levels of 18S mRNA in each sample. The optical density was standardized in relation to control values (taken as 1) and presented as fold increase. Bars in figures show means ± SE of three to four independent Northern blots.

Plasmids and templates. Mouse 18S is a 1.212-kb plasmid insert (Mouse DECAprobe template; Ambion, Austin, TX). The EcoRI-XbaI fragment of the murine LIF complementary DNA (cDNA) spanning the entire coding sequence of murine LIF (2–631 bp; GenBank accession no. A01690; provided by Dr. Tracy Willson, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) was cloned into a pcDNA3 vector, isolated, electrophoresed in 1.2% agarose gel, and extracted with QuiiaX II. Murine SOCS-3 cDNA (19–610 bp; GenBank accession no. U88328; 20-bp primers) was isolated in our laboratory by RT-PCR of murine pituitary mRNA (5). Before use as a template for random priming, the specificity of the RT-PCR product was verified by multiple-restriction

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enzyme analysis. The 0.6-kb fragment of murine POMC cDNA, encoding the 3’ half of exon 3 was kindly provided by Dr. Malcolm J. Low (Portland, OR). Probes were labeled by random priming with a Random Primer Labeling Kit (Stratagene, La Jolla, CA).

Relative RT-PCR. Total pituitary RNA was prepared as described, and, before the reverse transcription reaction, RNA samples were treated with DNase I (DNA-free DNase Treatment & Removal Reagents; Ambion) to eliminate DNA contamination. Total RNA (2.5 μg) was reverse transcribed into first-strand cDNA by use of a SuperScript Preamplification System (GIBCO-BRL) and random hexamers according to the manufacturer’s protocol. Subsequent PCR reactions were performed using a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT).

Relative levels of TNF-α, IL-1β, and IL-6 gene expression were assessed using Gene Specific Relative RT-PCR Kits (Ambion), containing primers for mouse TNF-α, IL-1β, or IL-6 and 18S as an internal control.

Multiplex RT-PCR for detection of TNF-α, IL-1β, and 18S expression was carried out for 32 cycles of amplification (94°C for 30 s, 59°C for 30 s, and 68°C for 30 s) after denaturation at 94°C for 2 min. For detection of IL-6 and 18S expression, cDNA was denaturated at 94°C for 2 min followed by 34 cycles consisting of 94°C for 30 s, 61°C for 30 s, and 72°C for 30 s. PCR cycles for each gene of interest were in the linear phase of amplification and were determined empirically. To decrease amplification efficiency of 18S cDNA to levels compatible with TNF-α, IL-1β, and IL-6 cDNA levels, 18S primers were mixed with 18S competitors (non-extendable 18S primers modified at 3’ end) at a ratio of 2:8. Amplification from cDNA samples produced products of the same size as positive control templates included in each kit, and the size of amplicons was as expected. Each PCR reaction contained 1 μl of RT product, 0.4 μM gene-specific primer, and 20 μl of PCR Master Mix (Qiagen, Valencia, CA). The RT-PCR product was analyzed by electrophoresis in 2% agarose gel. Gels were photographed under ultraviolet light using Polaroid 667 film (Polaroid, Cambridge, MA). To quantify RT-PCR product, reactions were spiked with 2.5 μCi of [α-32P]dCTP per tube, as described (44). Products were run in 6% sequencing gels (Sequagel System, National Diagnostic, Atlanta, GA), transferred to 3-MM paper, dried, and exposed to BioMax-MR X-ray film (Kodak, Rochester, NY). Each PCR reaction was repeated three to five times for every experiment. Relative mRNA levels of IL-1β, IL-6, and TNF-α in separate experiments were determined by densitometry using an Alphalmager 2000 normalized against levels of 18S mRNA in each sample. The values were expressed as arbitrary units relative to WT untreated or PBS-treated controls from the same experiment taken as one (44). Bars in figures show means ± SE of three to four independent experiments.

Statistical analysis. Data were analyzed using one-way analysis of variance within genotypes followed by nonparametric t-test (Mann-Whitney Test). A t-test was used for single comparisons between WT and LIFKO mice.

RESULTS

Six to seven control animals were injected with sterile PBS and killed 1, 4, or 8 h after injection. No changes were observed in pituitary LIF, TNF-α, IL-1β, IL-6, or SOCS-3 gene expression throughout the 8-h observation period. However, plasma corticosterone levels increased moderately (20%) 1 h after PBS injection, and by 4 h, corticosterone levels returned to normal. Therefore, with this nonspecific injection effect on plasma stress hormones levels taken into account and to limit the number of animals required, mice of both genotypes were injected with PBS and killed 1 h after treatment and used as controls for each time point.

Time course of plasma ACTH and corticosterone levels after LPS treatment. After intraperitoneal injection of 50 μg of LPS, pituitary LIF mRNA levels in WT mice were induced 9 ± 0.5-fold (P < 0.05) at 1 h and peaked (16 ± 2-fold, P < 0.05) 4 h after the inflammatory challenge (Fig. 1). The role of LIF in HPA axis activation was assessed in WT and LIFKO mice for up to 8 h after LPS injection. Plasma ACTH levels did not differ in WT and LIFKO control mice (135 ± 35 vs. 87 ± 28 pg/ml, respectively). In WT mice, ACTH levels increased at 1 h (from 135 ± 35 to 380 ± 72, not significant), peaked 4 h after injection (630 ± 76 pg/ml, P < 0.01 vs. controls), and remained elevated 8 h after LPS injection (480 ± 66 pg/ml, P < 0.05 vs. controls). In LIFKO animals, the time course of the ACTH response differed. Plasma ACTH levels peaked 1 h after LPS treatment (718 ± 85 pg/ml, P < 0.01 vs. controls) and
were actually higher than in WT animals (P < 0.05) at this time. However, by 4 h, levels had already dropped below those observed in WT mice (331 ± 56 vs. 630 ± 76 pg/ml, P < 0.5) and continued to decline 8 h after injection (Fig. 2). Baseline levels of plasma corticosterone were elevated in both genotypes after PBS treatment. Circulating corticosterone levels in WT mice rose markedly starting at 30 min (from 350 ± 118 in controls to 998 ± 121 ng/ml, P < 0.05) and continued to rise for up to 8 h after LPS treatment (1,420 ± 188 ng/ml, P < 0.01 vs. controls). In LIFKO mice, although plasma corticosterone was elevated above control levels at 1 h (659 ± 98 vs. 175 ± 54 ng/ml, P < 0.05), 4 h (770 ± 136 vs. 175 ± 54 ng/ml, P < 0.05), and 8 h (882 ± 101 vs. 175 ± 54 ng/ml, P < 0.05) after injection, this elevation was lower (P < 0.05) than in WT animals at the same times (Fig. 2).

**Pituitary cytokine gene expression.** Proinflammatory cytokines induced within the pituitary during the course of inflammation are involved in activating the HPA axis inflammatory response (37). A detectable transcript of pituitary IL-1β and TNF-α could be seen only after longer exposure of Northern blots (data not shown). Because of the low levels, IL-1β, IL-6, and TNF-α cytokine gene expression in WT and LIFKO animals were further analyzed by competitive RT-PCR. After LPS inoculation, pituitary IL-1β mRNA levels increased more abundantly in LIFKO mice at 1 h (81 ± 5 in LIFKO vs. 64 ± 7 in WT, P < 0.05) and 8 h (32 ± 1 in LIFKO vs. 18 ± 3 in WT, P < 0.05) after treatment. A similar pattern was noted for pituitary IL-6 gene expression, which was induced further in LIFKO mice 1 h after endotoxin treatment (25 ± 2 in LIFKO vs. 15 ± 3 in WT, P < 0.05) (Fig. 3A). TNF-α induction was also higher in LIFKO animals (211 ± 9 in LIFKO vs. 94 ± 3 in WT, P < 0.05) (Fig. 3B).

TNF-α is an early cytokine, readily detectable in plasma after an LPS challenge (21). To analyze mechanisms for inflammation-associated LIF induction, we examined whether pituitary LIF was stimulated by TNF. LIF gene expression was markedly induced 1 h after intraperitoneal injection of 0.5 μg of TNF-α (Fig. 4A). Because TNF-α stimulates IL-1 synthesis and release (2), pituitary IL-1β gene expression in WT and LIFKO was also analyzed. One hour after TNF inoculation, IL-1β was induced in both genotypes. However, in LIFKO mice, the increase of IL-1β mRNA levels was more robust (18 ± 3 in LIFKO vs. 10 ± 2 in WT, P < 0.05) (Fig. 4B).

**Pituitary SOCS-3 gene expression after LPS treatment.** SOCS-3 is an intracellular negative regulator of cytokine signaling in pituitary corticotrophs. Considering the strong induction of intrapituitary inflammatory cytokines observed in LIFKO mice, we examined pituitary SOCS-3 expression. In WT mice, pituitary SOCS-3 mRNA levels increased at 1 h (6 ± 0.6-fold, P < 0.05), peaked at 4 h (10 ± 1-fold, P < 0.05), and remained persistently elevated 8 h (9.1 ± 1-fold, P < 0.05) after LPS injection compared with control PBS-treated animals. In LIFKO mice, the time course of pituitary SOCS-3 induction was similar. Pituitary SOCS-3 mRNA levels increased at 1 h (9 ± 1-fold, P < 0.05), peaked at 4 h (15 ± 1.0-fold, P < 0.05), and remained higher at 8 h (13 ± 1-fold, P < 0.05). However, the overall increase of pituitary SOCS-3 gene expression was considerably stronger in LIF-deficient mice. Differences in SOCS-3 induction within the two experimental groups were greater at 4 h (15 ± 1.0-fold vs. 10 ± 1-fold, P < 0.05) and 8 h (13 ± 1-fold vs. 9 ± 1-fold, P < 0.05) after treatment (Fig. 5).

**Relationship between SOCS-3 and POMC gene expression in vitro.** SOCS-3 appeared to play a critical role in inhibiting cytokine-mediated POMC induction (5). To further investigate the physiological role of SOCS-3 in restricting pituitary POMC expression, levels of POMC and SOCS-3 mRNA abundance in the same experimental settings were examined in AtT20 mouse corticotrophs. IL-6 induces both SOCS-3 and POMC gene expression in a timely manner (5). In control cells treated with MM oligonucleotides, SOCS-3 was induced, whereas POMC was abrogated 1 h after IL-6 treatment. Conversely, pretreatment of AtT20 cells with SOCS-3 AS oligonucleotides suppressed SOCS-3 mRNA levels with subsequent increased POMC expression. AS also abrogated the SOCS-3 response to IL-6 (Fig. 6). The POMC/SOCS-3 relationship...
mRNA ratio derived from three independent experiments (Table 1) showed a negative relationship between POMC and SOCS-3 gene expression.

Inflammatory responses after LPS injection. The impact of HPA axis activation on the severity of inflammation in LIFKO animals was analyzed. Baseline ESR did not differ between the two genotypes. After LPS injection, acceleration of the ESR was noted at 1 h, and in LIFKO mice, endotoxin-induced ESR was higher compared with WT animals (22 ± 3 vs. 16 ± 1 mm, P < 0.05) at 16 h (Fig. 7).

Plasma levels of the acute-phase response SAA were determined before and after LPS injection. Baseline SAA levels in LIFKO mice were higher than in WT animals (8 ± 3 vs. 2 ± 0.1 μg/ml), although the difference was not significant. Thirty hours after LPS injection, SAA levels rose markedly in LIFKO mice but not in WT animals (38 ± 10 vs. 5 ± 2 μg/ml, P < 0.01) (Fig. 7).

DISCUSSION

We show here that LIF is an important component of the cytokine network regulating the neuroendocrine response to inflammation. Baseline pituitary LIF expression is very low in healthy, inflammation-free animals and is induced in response to endotoxin stimulation. Pituitary LIF gene activation could result from the stimulatory effects of cytokines produced during systemic inflammation. Our current in vivo results indicate that pituitary LIF induction is at least partly stimulated by TNF-α released in the course of endotoxic shock. Thus pituitary LIF mRNA levels increased significantly 1 h after TNF-α treatment. We recently
reported marked hypothalamic and modest pituitary LIF induction in animals with chronic local inflammation (15). These results are in contrast to the present finding that LIF is strongly induced in the pituitary after LPS treatment. LPS evokes acute inflammatory shock, resulting in high systemic levels of proinflammatory cytokines. LPS directly induces secretion of brain immunoregulatory cytokines that modulate their own pituitary and peripheral levels (31). The pituitary is exposed to circulating macrophages and peripheral cytokines, which may stimulate pituitary LIF expression in a paracrine or endocrine manner (20, 21, 37). In contrast, a local inflammatory process does not increase high cytokine levels. The results suggest that, in mice with local chronic inflammation, hypothalamic LIF is more likely induced via prolonged stimulation of visceral afferents originating at the site of inflammation.

When it is considered that LIF is a potent activator of pituitary POMC transcription and ACTH secretion (2, 7, 11, 14, 29), the induction of pituitary LIF gene expression could be an important component for HPA axis function during LPS-induced inflammatory stress. HPA responses were therefore compared in WT and LIFKO mice. Overall, LIF deficiency leads to blunting of pituitary SOCS-3 expression (Fig. 5A). Relative abundance of SOCS-3 mRNA was quantified by densitometry and normalized against levels of 18S rRNA in each sample. Optical density was standardized in relation to the control values (taken as 1) and shown as fold increase. Bars show means ± SE of 3 independent Northern blots. *Differs from control value at \( P < 0.05 \).

Table 1. Relationship between pituitary SOCS-3 and POMC expression

<table>
<thead>
<tr>
<th>Treatment</th>
<th>POMC/SOCS-3 Ratio</th>
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<tr>
<td>Control</td>
<td>1 ± 0.04</td>
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<tr>
<td>AS (anti-SOCS-3 oligonucleotides)</td>
<td>1.3 ± 0.26</td>
</tr>
<tr>
<td>MM (mismatched oligonucleotides)</td>
<td>0.8 ± 0.12</td>
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<tr>
<td>AS + IL-6</td>
<td>0.7 ± 0.04</td>
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<tr>
<td>MM + IL-6</td>
<td>0.45 ± 0.06*</td>
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Ratios of proopiomelanocortin (POMC) to suppressor of cytokine signaling (SOCS-3) are expressed as fold induction over untreated controls and are means ± SE of 3 independent experiments. ATt20 cells were pretreated with antisense (AS) or mismatch (MM) for 36 h and then treated with 25 nM murine interleukin (IL)-6 for 1 h. Relative abundance of SOCS-3 mRNA was analyzed by Northern blot, determined by densitometry, and normalized to levels of POMC mRNA in the same sample. * \( P < 0.05 \) vs. control.
of the ACTH and corticosterone responses to inflammatory stress.

HPA stress response and glucocorticoids restrict the inflammatory process mostly by inhibiting synthesis and release of proinflammatory cytokines (32, 33). A variety of hypothalamic and pituitary cytokines and their receptors are constitutively expressed and play an important role in modulating pituitary hormone secretion (16, 25, 29, 37). In these experiments, pituitary IL-1β, IL-6, and TNF-α expression increased in response to inflammatory stress. Pituitary cytokines are to some extent also a component of the peripheral cytokine pool. We therefore cannot exclude that circulating stimulated immune cells (e.g., macrophages) residing in the pituitary may contribute to the observed levels of pituitary cytokine messenger RNA detected by supersensitive RT-PCR. Nevertheless, this would still reflect disordered responses of LIFKO animals to inflammation. Levels of cytokine expression in untreated and PBS-treated mice of both genotypes are very low, at the limit of RT-PCR sensitivity. We therefore chose to compare only induced levels of cytokine expression. Overall, mRNA levels for pituitary cytokines were higher in animals with LIF deficiency.

Higher levels of pituitary cytokines after LPS and TNF-α administration in the absence of LIF suggest that LIF is implicated in the complex synergistic relations between circulating cytokines. Thus an acute inflammatory response during endotoxemia is mediated by induction of proinflammatory cytokines such as TNF-α (19, 28). Subsequently, TNF-α induces IL-1β (18), which in turn, stimulates IL-6 synthesis and secretion (17). LIF decreased serum TNF-α concentrations in vivo (38) and in vitro (43). Such an anti-inflammatory action of LIF is in accord with results demonstrating a protective effect of exogenous LIF in septic shock (40) and local inflammation (8). Absence of LIF leads to TNF-α overproduction with subsequent increased IL-1β and IL-6 expression in LIFKO animals.

It is not clear whether LIF deficiency per se or lower corticosterone levels result in higher cytokine produc-

Fig. 7. Inflammatory response in WT and LIFKO mice after LPS injection. Erythrocyte sedimentation rate (ESR) was measured using Wintrobe 3 × 115-mm tubes. WT and LIFKO mice (n = 8 per group) were killed untreated or 8 h after LPS injection, and measurements were made 16 h later. *P < 0.05 for LPS-treated vs. untreated mice. Serum amyloid A (SAA) was measured in 20 μl of serum from WT and LIFKO mice (n = 8 per group) killed at 0 (controls), 8, or 30 h after LPS injection. *P < 0.05 for WT vs. LIFKO mice.

Fig. 8. Schema illustrating complex relationships between LIF, other proinflammatory cytokines, and SOCS-3 in the pituitary.
tion in knockout animals. High levels of cytokines observed especially after 1-h treatment could be responsible for the peak plasma ACTH (3, 37) noted in LIFKO mice. We did not observe a corresponding corticosterone increase, likely due to the experiment timing. Thus an absence of LIF, but not a lower corticosterone, resulted in enhanced proinflammatory cytokine production at the beginning of the inflammatory process. However, we cannot exclude that lower plasma corticosterone responses in LIFKO mice may contribute to later stimulation of IL-1β 8 h after LPS treatment.

Increased pituitary cytokine induction may lead to pituitary SOCS-3 overexpression demonstrated in LIFKO animals. In these LIF-deficient animals, SOCS-3 was induced earlier, within 30 min, and remained elevated for up to 8 h after treatment. By inducing the JAK/STAT-3 pathway, proinflammatory cytokines stimulate both POMC and SOCS-3 transcription. SOCS-3, acting via an intracellular autocrine loop, negatively regulates cytokine signaling (4, 5) by inhibiting JAK kinase activity (10, 27). At the same time, SOCS-3 attenuates stimulatory effects of cytokines on pituitary POMC (5). These observations suggest that cytokine-induced SOCS-3 may mediate a pituitary response to inflammation. To test this hypothesis, we employed mouse pituitary corticotroph AtT20 cells treated with IL-6 that timely stimulates both SOCS-3 and POMC expression. In control cells treated with MM oligonucleotides, SOCS-3 expression induced by IL-6 was high when POMC expression was low. Treatment cells with SOCS-3 AS oligonucleotides resulted in a decrease of IL-6-induced SOCS-3 expression, whereas expression of POMC became induced. Statistical analysis shows a negative relationship between SOCS-3 and POMC gene expression. The results support the involvement of SOCS in regulation of the HPA axis. In the course of septic shock, a number of cytokines acting in synergy potentiate pituitary ACTH (22). The presence of rapid cytokine-inducible mechanisms restricting POMC overexpression could be an important step in preserving homeostasis. We earlier demonstrated attenuated POMC gene expression and ACTH secretion in LIFKO mice (1, 6, 14, 15). Simultaneously, SOCS-3 overexpression in response to LPS in LIFKO mice impacts on HPA axis functioning. Thus LIF deficiency may account for two different mechanisms leading to lowered HPA axis inflammatory responses.

Lower circulating ACTH and glucocorticoid levels, excessive production of pituitary cytokines, and SOCS-3 overexpression, which may reinforce suppression of the HPA inflammatory response, leads to increased inflammatory activity in LIFKO mice. This is reflected by accelerated ESR and increased SAA levels in these animals. SAA, an important acute-phase protein, is a sensitive indicator of inflammation (24), simultaneously providing enhanced protection from microorganisms (36). gp130 Cytokines induce SAA in an overlapping or redundant fashion when administered alone or in combination with IL-1. (36). The high levels of SAA observed in LIFKO mice could result from a strong inflammatory reaction occurring in the absence of LIF. Interestingly, even in untreated LIFKO animals, baseline SAA levels tend to be higher than in WT mice. This could reflect a compensatory elevation of other proinflammatory cytokines.

In summary, we demonstrate that pituitary LIF suppresses pituitary cytokine levels and activates the ACTH responses to acute inflammation. The negative correlation between pituitary SOCS and POMC expression could result in lowering of HPA function and further propagation of the inflammatory process. Figure 8 depicts complex relationships between LIF, other proinflammatory cytokines, and SOCS-3 in the pituitary. The multifaceted cascade of cellular and molecular events required for systemic homeostasis in response to infection or inflammation thus invoke both LIF and SOCS-3 in the neuroimmunoendocrine interface.

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REFERENCES
12. Bousquet C, Susini C, and Melmed S. Inhibitory roles for SHP-1 and SOCS-3 following pituitary proopiomelanocortin
LIF AND SOCS-3 IN HPA AXIS INFLAMMATORY RESPONSE


Sapolsky RM, Romero LM, and Munk AU. How do glucocor-


Wang Z, Ren S-G, and Melmed S. Hypothalamic and pituitary leukemia inhibitory factor gene expression in vivo: a novel endo-


