Leukemia inhibitory factor regulates glucocorticoid receptor expression in the hypothalamic-pituitary-adrenal axis

Anastasia Kariagina, Svetlana Zonis, Mahta Afkhami, Dmitry Romanenko, and Vera Chesnokova

Cedars-Sinai Medical Center and David Geffen School of Medicine at University of California, Los Angeles, California

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Kariagina, Anastasia, Svetlana Zonis, Mahta Afkhami, Dmitry Romanenko, and Vera Chesnokova. Leukemia inhibitory factor regulates glucocorticoid receptor expression in the hypothalamic-pituitary-adrenal axis. Am J Physiol Endocrinol Metab 289: E857–E863, 2005. First published June 28, 2005; doi:10.1152/ajpendo.00577.2004.—Leukemia inhibitory factor (LIF) is a pleiotropic cytokine belonging to the gp130 family. LIF is induced peripherally and within the brain during inflammatory or chronic autoimmune diseases and is a potent stimulator of the hypothalamic-pituitary-adrenal (HPA) axis. Here we investigated the role of LIF in mediating glucocorticoid receptor (GR) expression in the HPA axis. LIF treatment (3 μg/mouse, ip) markedly decreased GR mRNA levels in murine hypothalamus (5-fold, P < 0.01) and pituitary (1.7-fold, P < 0.01) and downregulated GR protein levels. LIF decreased GR expression in murine corticotroph cell line AtT20 within 2 h, and this effect was sustained for 8 h after treatment. LIF-induced GR mRNA reduction was abrogated in AtT20 cells overexpressing dominant-negative mutants of STAT3, indicating that intact JAK-STAT signaling is required to mediate LIF effects on GR expression. Conversely, mice with LIF deficiency exhibited increased GR mRNA levels in the hypothalamus and pituitary (3.5- and 3.5-fold, respectively; P < 0.01 for both) and increased GR protein expression when compared with wild-type littermates. The suppressive effects of dexamethasone on GR were more pronounced in LIF-null animals. These data suggest that LIF maintains the HPA axis by decreasing GR expression and raising the possibility that LIF might contribute to the development of central glucocorticoid resistance during inflammation.

LIF-null mice; AtT20 cells

GLUCOCORTICOIDS HAVE A WIDE RANGE of functions in regulating energy homeostasis, responses to stress, immune defenses, behavioral responses, and brain activity. Glucocorticoids also play a major role in negatively regulating the functioning of the hypothalamic-pituitary-adrenal (HPA) axis. Glucocorticoids signal by binding to two distinct cytosolic receptors, the mineralocorticoid receptor and the ubiquitous glucocorticoid receptor (GR) (3, 13, 29). GR-mediated signaling suppresses the HPA axis at multiple levels. In the pituitary, GR signaling inhibits expression of proopiomelanocortin (POMC) by directly binding to the glucocorticoid response element on the POMC promoter (27). However, most GR-mediated signaling indirectly regulates target genes by influencing intermediary transcription factors such as Jun-B, NF-κB, STAT3, and STAT5 (5, 12, 20, 39).

Glucocorticoids can suppress inflammation by inhibiting expression of proinflammatory cytokines through a number of mechanisms (3, 29). Conversely, inflammation is frequently accompanied by impaired central or local tissue-specific GR function (11, 22, 26, 36, 37). For example, attenuation of GR-mediated signaling occurs in peripheral lymphocytes in patients with asthma (21, 22) and rheumatoid arthritis (11). Proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) that are typically expressed in such conditions locally attenuate glucocorticoid effects in target tissues (17, 18, 26) by reducing the affinity of the GR for its ligand (14, 18), promoting expression of a dominant-negative GRβ isofrom (36), or interfering with GR shunting from the cytoplasm to the nucleus (26).

Leukemia inhibitory factor (LIF) is a proinflammatory cytokine of the gp130 family that is induced in many inflammatory diseases (8, 10). LIF binds to a heterodimeric complex that includes the specific LIF receptor (LIFR) and a promiscuous gp130 subunit that is shared among all members of the gp130 cytokine family. Upon ligand binding, the LIFR-gp130 complex can activate the JAK-STAT pathway, the MAPK pathway, or both, depending on the cell type (2). Previous studies from our laboratory (1) showed that LIF was a strong stimulator of the HPA axis. LIF potently induces POMC expression and ACTH secretion, and LIF-deficient animals exhibit a shortened ACTH response to psychological and inflammatory stress (8–10). Transgenic mice overexpressing pituitary LIF show not only elevated ACTH and corticosterone levels but also reduced sensitivity of pituitary POMC to the inhibitory action of dexamethasone (DEX) (38). These data suggest that LIF may attenuate negative feedback regulation of the HPA axis. The goal of the present study was to test this possibility.

Here, we report that LIF treatment reduced GR mRNA and protein expression in the HPA axis. In contrast, mice lacking LIF exhibited elevated GR mRNA and protein levels in the hypothalamus and pituitary. LIF caused a rapid decrease of GR expression in AtT20 murine corticotroph cells in a range of doses, and these effects were abrogated in AtT20 cells overexpressing dominant-negative forms of STAT3. Our data are most consistent with the interpretation that LIF is an essential mediator of HPA axis GR expression and may contribute to impaired negative feedback regulation of the HPA axis. Furthermore, although several signaling options are available to LIF, the effects of LIF on GR expression appear to be mediated predominantly or perhaps solely through the JAK-STAT signaling pathway.

MATERIALS AND METHODS

Experimental animals. C57Bl/6J female mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Lif−/− mice, originally

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generated by Dr. Colin L. Stewart (Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ) on C57BL/6J and DBA/2 heterozygous background, were back-crossed to C57BL/6J mice for at least five generations before being used in our experiments. LIF-null mice as well as wild-type littermates were obtained through breeding of heterozygous Lif<sup>+/−</sup> females and males. Genotype of pups was determined by PCR analysis of tail DNA (8). Five animals were housed per cage with free access to water and food at a standard 12:12-h dark-light cycle. The Institutional Animal Care and Use Committee at Cedars-Sinai approved all experimental procedures before we began our investigation.

**In vivo treatments.** Mice were injected intraperitoneally with recombinant murine LIF (3 μg/mouse; Chemicon International, Temecula, CA) in 200 μl of normal saline. Control animals received saline only. All mice were killed 3 h after injection. DEX (Sigma) was dissolved in 100% ethanol, diluted to a final concentration of 5 mg in a 5% ethanol–normal saline solution, and injected intraperitoneally (total injected volume 200 μl). Control animals were injected with the same volume of 6% ethanol in normal saline. Three hours after treatment, mice were killed, and the hypothalamus, pituitary, and liver were dissected, snap-frozen in liquid nitrogen, and stored at −80°C until RNA or protein extraction.

**Cell culture and treatments.** Murine corticotroph AtT20 cells (American Type Culture Collection, Rockville, MD) were grown in low-glucose-DMEM supplemented with 100 U/ml streptomycin, 100 U/ml penicillin, and 10% fetal calf serum (Mediatech, Herndon, VA). Before experimental treatments, cells were kept in serum-free medium for 18 h. Cells were treated with recombinant murine LIF (2–50 ng/ml), TNF-α (10 ng/ml), IL-1β (1 ng/ml), or IL-6 (10 ng/ml; all from R&D Systems, Minneapolis, MN). Two distinct types of dominant-negative STAT3 mutant constructs were used. The first (STATF) included a phenylalanine substitution at a tyrosine residue near the carboxyl-terminal residue (Tyr<sup>705</sup>). The second dominant-negative STAT3 construct (STATD) contained two alanine substitutions at positions important for STAT3 DNA binding (Glu<sup>134</sup> and Glu<sup>137</sup>). These mutants were transfected into AtT20 cells, and transfectants were selected with G418 (6).

**Northern blot analysis.** Total RNA was extracted from tissues of AtT20 cells using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol, and quantified spectrophotometrically. Total RNA (10 μg) was resolved on denaturing 1% agarose gel, transferred to a Hybond N+ membrane, and hybridized with a 32P-labeled fragment of murine GR (obtained by PCR, GenBank accession no. X04435) or 18S RNA (loading control; Decatemplate, Ambion, Austin, TX). Developed films were scanned, and densitometry was performed using Kodak imaging software.

**Real-time PCR.** Total RNA (2.5 μg) was treated with deoxyribonuclease I (DNase I; Ambion, Austin, TX) and reverse transcribed using OmniScript reverse transcriptase (Qiagen, Valencia, CA). Real-time PCR reactions were performed using an iCycler thermal cycler with an optical module (Bio-Rad, Hercules, CA) and a fluorescent reporter dye (SYBR Green I; Molecular Probes, Eugene, OR) as described (19). Primers were as follows: 18S sense, 5′-gaaagctgcaacagcataccag-3′; antisense, 5′-ctccatattgggttttcgc-3′ (product size 155 bp, melting temperature Tm = 88°C); GR sense, 5′-ggaaatatttgtgcactaaggac-3′; GR antisense, 5′-cggaaatattgtgcactaaggac-3′ (product size 150 bp, Tm = 89°C). The relative quantity of each gene in experimental samples was determined from the corresponding standard curve and normalized to 18S. Levels of mRNA are presented as arbitrary units referenced to the wild-type control, which was assigned an arbitrary value of 1.0 for comparative purposes.

**Western blot analysis.** Total protein as well as cytosolic and nuclear fractions were isolated from hypothalamus, pituitary, and AtT20 cells as previously described (30). Proteins were quantitated using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Twenty micrograms per lane were resolved on 8% polyacrylamide gel (Express Gels; ISC BioExpress, Kaysville, UT) and transferred to an Immobilon membrane (Millipore, Bedford, MA). After blocking with 5% milk in a buffer, membranes were incubated with anti-GR antibody (1:5,000 dilution; Affinity Bioreagents, Golden, CO) and anti-β-actin antibody (1:2,000 dilution; Sigma, St. Louis, MO). After incubation with secondary antibodies (Amersham, Piscataway, NJ), membranes were treated with enhanced chemiluminescence (ECL) or ECL plus reagents (Amersham) and exposed on X-ray film. Developed films were scanned, and densitometry was performed using Kodak imaging software.

**Statistical analysis.** Data were analyzed by ANOVA, followed by a nonparametric Mann-Whitney test or Student’s t-test. All data are presented as means ± SE.

**RESULTS**

**LIF reduces GR expression in vivo.** To address the question of whether LIF contributes to negative feedback regulation of the HPA axis, wild-type mice were injected with recombinant murine LIF, and GR protein and mRNA levels were analyzed in the hypothalamus and pituitary. Three hours after LIF treatment, GR protein levels in whole cell lysate, cytoplasmic fractions, and nuclear fractions were all decreased in the hypothalamus (Fig. 1, A and C). In the pituitary the diminished GR protein levels were even more evident (Fig. 1, B and D). We also observed a reduction in GR mRNA levels in the hypothalamus and pituitary (P < 0.01 for both) compared with control mice injected with saline (Fig. 1, E and F).

**LIF downregulates GR expression in vitro.** We previously reported that LIF strongly increased plasma corticosterone levels in vivo (8). It is possible that the reduction in GR mRNA expression in the hypothalamus and pituitary that we observed might have been secondary to increased plasma levels of corticosterone. To evaluate this possibility, we treated mouse AtT20 pituitary corticotrophs with LIF (2–50 ng/ml) for 4 h. LIF in a range of doses diminished GR expression (Fig. 2A). Treatment with LIF (10 ng/ml) reduced GR mRNA levels in AtT20 cells beginning after 2 h of treatment, was sustained for ≥8 h, and by 24 h GR expression had returned to baseline levels (Fig. 2B).

To address the question of specificity of LIF action, we treated AtT20 cells with recombinant murine TNF-α, IL-1β, or IL-6 (another gp130 cytokine). None of these cytokines altered GR mRNA expression 2–24 h after treatment (Fig. 2, C and D, and data not shown). These results support the interpretation that LIF specifically and directly reduces GR mRNA expression and that this decrease may not be mediated by glucocorticoids.

To determine whether the LIF-induced decrease in GR mRNA levels was associated with a parallel decrease in GR protein levels, we performed Western blotting of whole cell lysates and cytoplasmic and nuclear fractions of AtT20 cells treated with 10 ng/ml LIF. No changes in GR protein levels were noted in whole cell lysates during the course of the experiment (Fig. 3A). The relative abundance of GR in cytosolic and nuclear fractions was quantified by densitometry and normalized against levels of β-actin in each sample. LIF treatment for 2 h markedly decreased cytosolic GR protein levels (P < 0.05, Fig. 3B), whereas the nuclear fraction of GR protein was increased (P < 0.05, Fig. 3C), consistent with the interpretation that LIF induces GR translocation from the cytoplasm to the nucleus. The results show that by 8 h after treatment, GR protein levels returned to baseline in both...
cytoplasm and nucleus (Fig. 3, D and E). Decreased cytoplasmic GR expression 24 h after treatment ($P < 0.05$ compared with control) can be attributed to the delayed effects of GR mRNA decline in LIF-treated cells (Fig. 2). These results suggest that LIF might be also involved in regulating nuclear-cytoplasmic shuttling of GR.

**LIF effects on GR expression are mediated by the JAK-STAT signaling pathway.** In mouse corticotrophs, LIF signaling utilizes the JAK-STAT3 signaling cascade (6). JAK-STAT signaling in AtT20 cells was inhibited by stably transfecting wild-type STAT3 (STATW, control) or a dominant-negative STAT3 with a mutation on either the phosphorylation site

![Figure 1](http://ajpendo.physiology.org/DownloadedFrom/10.220.33.3.on.June.29.2017)

**Fig. 1.** Effects of LIF injection on glucocorticoid receptor (GR) and protein mRNA levels in wild-type mice. Animals were killed 3 h after treatment. Western blot analysis of GR in whole cell lysate (A and B) cytosolic (cyto) and nuclear (nucl) fractions in hypothalamus (C) and pituitary (D), and real-time PCR measurements of hypothalamic (E) and pituitary (F) GR mRNA levels. Results are expressed in arbitrary units. Cont, PBS injection. Values are means ± SE of ≥3 replicate measurements. Data are representative of 3 experiments; $n = 8–10$ animals/group. **$P < 0.01$ compared with control.

![Figure 2](http://ajpendo.physiology.org/DownloadedFrom/10.220.33.3.on.June.29.2017)

**Fig. 2.** Effects of LIF on GR mRNA levels in AtT20 cells. A: cells were treated with different doses of LIF and harvested 4 h later. Experiment was performed twice with similar results, and a representative blot is shown. B: cells were treated with LIF (10 ng/ml) and harvested at indicated time points. A representative blot is shown. Relative abundance of GR mRNA was quantified by densitometry and normalized against levels of 18S mRNA in each sample. Optical density was standardized in relation to control values (taken as 1) and shown as fold increase. Bars show means ± SE of 3 independent Northern blots. **$P < 0.01$ compared with control. Cells were treated with 10 ng/ml TNF-α (C) or IL-6 (D) and harvested at indicated time points. Experiment was performed twice with similar results.
STATF) or the DNA-binding domain (STATD) (6). After treatment of stably transfected AtT20 cells with LIF, Northern blot analysis showed decreased GR mRNA levels in cells expressing STATW as expected. In contrast, LIF treatment of cells that overexpressed either of the dominant-negative constructs failed to reduce GR mRNA expression (Fig. 4). These results indicate that LIF treatment directly inhibits GR expression via JAK-STAT pathway stimulation, and thus provide support for the interpretation that LIF regulates GR expression by a mechanism that depends upon intact JAK-STAT signaling.

GR levels in LIF-null mice. To determine the physiological role of LIF in regulating GR in the HPA axis, we examined expression of GR in Lif−/− mice. Compared with wild-type mice, GR mRNA levels were elevated in the hypothalamus (3.6-fold, \( P < 0.01 \)) and pituitary (3.6-fold, \( P < 0.01 \)) but not in the liver of Lif−/− mice (Fig. 5A). Whole cell GR protein levels were also increased in both the hypothalamus and pituitary of Lif−/− animals relative to wild-type littermates (Fig. 5, B and C). These findings demonstrate that genetic deficiency of LIF results in augmented GR mRNA and protein levels in the HPA axis, and thus support the in vivo relevance of results obtained from our cell culture studies.

GR mRNA autoregulation in Lif−/− mice. To explore whether LIF was involved in GR autoregulation, we treated LIF-null and wild-type mice with DEX, killed them 2 h later, and determined GR mRNA levels in the hypothalamus, pituitary, and liver. DEX suppression of hypothalamic GR mRNA levels was more pronounced in Lif−/− than in wild-type mice (94% inhibition in Lif−/− mice vs. 75% in wild-type animals, \( P < 0.01 \)). Similarly, in the pituitary, DEX lowered GR mRNA expression (89% inhibition in Lif-deficient mice and only 46% inhibition in wild-type animals, \( P < 0.01 \)). Thus the HPA axis in Lif−/− animals appeared to be more sensitive to the suppressive action of DEX. At the same time, mouse genotype

Fig. 3. Effects of LIF on GR protein levels in AtT20 cells. Western blot analysis of GR protein levels in whole cell lysate (A), cytosolic (B), and nuclear fractions (C) of AtT20 cells treated with LIF (10 ng/ml) and harvested at indicated time points. Relative abundance of GR in cytosolic (D) and nuclear fractions (E) was quantified by densitometry and normalized against levels of β-actin 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 Western blots. *\( P < 0.05 \) compared with control. C, untreated.

Fig. 4. Effects of LIF on GR expression in dominant-negative STAT3 mutant cells. A: Northern blot analysis of GR mRNA in regular AtT20 cells and AtT20 cells overexpressing dominant-negative STAT3 and treated with LIF (10 ng/ml). STATW, cells overexpressing wild-type STAT3; STATD, cells overexpressing STAT3 mutated on DNA binding site; STATF, cells overexpressing STAT3 mutated on phosphorylation site; unt, untreated. A representative blot is shown. B: relative abundance of GR mRNA was quantified by densitometry and normalized against levels of 18S mRNA 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. *\( P < 0.05 \) compared with untreated AtT20 cells. §\( P < 0.05 \) compared with untreated cells overexpressing STATW.
Impaired GR sensitivity to corticosteroids during stress and inflammation may be attributed to multiple and diverse mechanisms, but the details are not fully understood on a molecular level. LIF is expressed in the hippocampus, hypothalamus, and pituitary (8, 10, 33) and is markedly elevated in response to acute and chronic inflammation (7, 9) and injury (4). Furthermore, plasma LIF levels correlate with the disease severity (34). LIF stimulates the HPA axis (1) and induces POMC expression and ACTH secretion, and LIF-deficient animals exhibit a shortened ACTH response to stress (8–10). Transgenic mice overexpressing pituitary LIF exhibit elevated ACTH and corticosterone levels and reduced sensitivity of pituitary POMC to DEX inhibition (38).

Here, we show that LIF attenuates the HPA axis negative feedback response by inhibiting GR expression. Treatment of wild-type mice with LIF reduces GR protein levels in the hypothalamus and pituitary, in whole cell lysates, and in both cytoplasmic and nuclear fractions. Hypothalamic and pituitary GR mRNA expression was also decreased 3 h after LIF injection. Accordingly, Lif−/− mice manifested increased levels of GR mRNA and protein in the hypothalamus and pituitary. Experiments in vitro confirmed a direct negative effect of LIF on GR expression. In mouse corticotrophs, GR mRNA levels were decreased for 2–8 h after LIF treatment and returned to baseline by 24 h. Inhibition of GR expression by LIF was mediated by signaling, utilizing the JAK-STAT pathway, specifically STAT3.

Recent studies have revealed that proinflammatory cytokines can influence the expression and function of GR. For example, treatment with cytokines or a cytokine inducer (LPS) alters GR expression in a number of cells and tissues, including T cells (18), monocyte/macrophages (14, 15), lung (31), and liver (16). However, in our experiments, the proinflammatory cytokine IL-6 (which belongs to the same gp130 cytokine family as LIF) did not change GR expression in AtT20 cells. Also, in contrast with a previous report that TNF-α and IL-1β could induce the GRβ isoform and negatively regulate GR function in human fibroblasts (36), our data showed that TNF-α or IL-1β treatment did not affect GR expression in AtT20 cells, perhaps because of the absence of the GRβ isoform in mouse corticotrophs (25). The explanation for these discrepancies is uncertain, but it should be noted that regulation of GR transcription appears to be under the control of several tissue-specific promoters (23). Hence, it is possible that different cytokines may have tissue-specific effects on GR expression and function.

Our data show that, in vitro, LIF alters subcellular localization but not overall cellular levels of GR. GR exists primarily in the cytoplasm but can translocate to the nucleus upon activation, where it binds to hormone response elements or interacts with various transcription factors. The amount of GR protein in the cytosolic fraction of AtT20 cells paralleled the decline in GR mRNA that occurred 2 h after LIF treatment, yet simultaneously, nuclear GR protein levels were increased. LIF-induced GR nuclear translocation decreases GR mRNA levels. Thus the GR receptor can recognize a specific binding sequence within the receptor cDNA and downregulate its own expression (35). The decreased cytoplasmic GR protein levels observed in AtT20 cells 24 h after treatment probably resulted from a preceding decrease in GR gene transcription. Therefore, it seems likely that GR protein levels may decline at a later time after LIF treatment.

LIF treatment markedly reduced GR mRNA levels both in vivo and in vitro. However, whereas in vivo GR protein levels decreased, in vitro LIF caused redistribution of GR from the cytoplasm to the nucleus. These discrepancies can be explained by the effects of high plasma levels of corticosterone observed in mice after LIF injection (8, 10). In addition to LIF effects, induced corticosterone levels can not only further diminish GR gene transcription (28) but also decrease the receptor half-life.
(24) by ubiquitin-dependent proteasomal degradation (32) in both the hypothalamus and the pituitary.

Consistent with the results obtained after LIF treatment, \( {LIF}^{−/−} \) animals demonstrated markedly increased GR mRNA and protein levels in the hypothalamus and pituitary relative to wild-type littermates. Thus, whereas LIF treatment in vitro resulted in a redistribution of GR, genetically induced LIF deficiency led to constitutively increased cellular GR protein levels as a consequence of induced GR mRNA expression in LIF-deficient animals. Importantly, the difference in GR expression between LIF-null and wild-type mice appears to be attributed to the HPA axis, since there was no difference in liver GR mRNA levels between LIF-null and wild-type mice.

Taken together, our studies demonstrate that LIF negatively regulates GR expression in the HPA axis under basal conditions. These data also suggest that LIF not only stimulates acute pituitary POMC induction and ACTH release (8–10) but that by suppressing GR expression LIF may also promote sustained HPA axis activation. Short-term inhibition of GR expression observed in pituitary cells may prolong the ACTH response to inflammatory stimuli and thus limit immune activation. Although our study did not involve experimental models of inflammatory diseases, our results suggest that LIF may contribute to the development of short-term central glucocorticoid resistance during inflammation by suppressing GR expression in the HPA axis. An important goal of future studies will be to directly test this hypothesis in appropriate animal models of inflammatory disease, and our data here now provide a conceptual foundation to pursue such studies.

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