Constitutive activation of STAT-3 and downregulation of SOCS-3 expression induced by adrenalectomy

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Constitutive activation of STAT-3 and downregulation of SOCS-3 expression induced by adrenalectomy. Am J Physiol Regulatory Integrative Comp Physiol 281: R2048–R2058, 2001.—Removal of adrenal steroids by adrenalectomy (ADX) slows or reverses the development of many forms of obesity in rodents, including those that are leptin or leptin receptor deficient. Obesity is associated with hyperleptinemia and leptin resistance. We hypothesized that glucocorticoids impair leptin receptor signaling and that removal thereof would activate the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling pathway. The inhibitory effect of leptin (2.5 μg iv) on food intake was enhanced in ADX rats. A combination of ribonuclease protection assays, RT-PCR, Western blots, and mobility shift assays was used to evaluate the leptin signaling pathway in whole hypothalami from sham-operated, ADX and corticosterone-replaced ADX (ADX-R) Sprague-Dawley rats that were treated acutely with either saline vehicle or leptin intracerebroventricularly. ADX increased the expression of leptin receptor mRNA, increased STAT-3 mRNA and protein levels, induced constitutive STAT-3 phosphorylation and DNA binding activity, and also reduced suppressor of cytokine signaling-3 (SOCS-3) mRNA and protein levels. ADX and leptin treatment increased STAT-3 phosphorylation, but with no concomitant increase in DNA binding activity. Leptin and ADX treatment did not further decrease NPY mRNA expression; their combination increased NPY mRNA expression. Corticosterone supplementation of ADX rats partially reversed many of these effects. In conclusion, ADX through activation of STAT-3 and inhibition of SOCS-3 activates the JAK-STAT signaling pathway. These effects most probably explain the ability to prevent the development of obesity by removal of adrenal steroids.

Obesity, through its associated comorbidities, is an escalating health problem throughout the world. Much of our knowledge of the mechanisms that contribute to the development of obesity comes from studies of animal models. Adrenalectomy (ADX) prevents the development of many forms of rodent obesity, including obesity in rodents that have a defect in the leptin signaling pathway. The mechanism through which this response is mediated remains unclear but reflects the removal of adrenal glucocorticoids, because glucocorticoid replacement of ADX animals restores fat deposition and obesity (4, 6, 22, 50), and blockage of type II glucocorticoid receptor with the antagonist RU-486 inhibits development of both genetic and dietary obesity (35, 44).

Leptin, a hormone secreted by the adipose tissue, plays an important role in the regulation of energy balance, providing a signal to the central nervous system on the levels of triglyceride stores (23, 24, 27). Glucocorticoids and insulin stimulate leptin gene expression in the adipose tissue and leptin protein secretion into the circulation (15, 53). Administration of glucocorticoids increases food intake in rodents, overriding the effect of increased endogenous leptin expression and secretion. These contrasting effects of glucocorticoids may suggest that glucocorticoids either reduce sensitivity to leptin or oppose leptin action through independent pathways. Indeed, an increase in response to leptin has been described in ADX rats by Zakrzewska and colleagues (58). Corticotropin-releasing hormone (CRH), which suppresses food intake, is negatively regulated by glucocorticoids. However, CRH deficiency after ADX did not affect food intake and body weight, indicating that factors other than or in addition to CRH are important in mediating food intake responses after ADX (31).

The leptin receptor (OBR) is a single membrane-spanning receptor that is similar to the class I cytokine receptor family (55). The majority of the transcripts in most tissues is those encoding the short forms of the receptor (OBR-S) (25), and the transcript that encodes the long form (OBR-L) is less abundant except in the hypothalamus. Within the hypothalamus, the OBR-L isoform is expressed in regions that are thought to control food intake and body weight, namely, the arcuate nuclei (Arc), ventromedial nuclei, and paraventricular nuclei (41). The functions of the long and short

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intracellular domains of the leptin receptor are currently being defined. The short isoforms play a major role in transporting leptin from the blood into the brain or for clearance (12). The long OBR isoform provides intracellular signaling by acting through the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway via the STAT-3 protein in the hypothalamus (57). The JAK proteins are associated with the receptor intracellular domain, where they phosphorylate tyrosine residues of the receptor upon ligand binding. The phosphorylated receptor then provides the docking site for STAT proteins, which are also tyrosine phosphorylated upon binding the phosphorylated receptor. The activated STAT proteins then dimerize and translocate to the nucleus to stimulate gene transcription (14).

Recently, a family of cytokine-induced cytokine signaling inhibitors has been described and it includes eight members, namely the cytokine-inducible SH2 proteins (CIS) and suppressor of cytokine signaling (SOCS)-1 to 7 (29). The expression of SOCS proteins is induced by various cytokines, and, once expressed, SOCS proteins downregulate JAK-STAT pathways and hence modulate the biological response. Peripheral administration of leptin to ob/ob mice rapidly induced SOCS-3 mRNA in hypothalamus, but not in db/db mice (3, 20). This leptin-dependent increase of SOCS-3 mRNA was seen in areas of the hypothalamus expressing high levels of the OBR-L. Furthermore, SOCS-3 was shown to block leptin-induced signal transduction in mammalian cell lines. The expression of SOCS-3 mRNA in the Arc and dorsomedial nuclei is increased in agouti mice, an obesity model of leptin resistance. This indicated that SOCS-3 is a leptin-inducible inhibitor of leptin signaling and suggests that SOCS-3 may also be a mediator of leptin resistance in obesity (3).

ADX has been shown to increase sensitivity to leptin (58). We hypothesized that this increase in leptin sensitivity after ADX could be mediated through several mechanisms, including direct effects on expression of the leptin receptor, changes in activation of the JAK-STAT pathway, or expression of SOCS-3 signaling or through direct effects on the neuropeptide genes that modulate the effects of leptin on energy balance. To determine if glucocorticoids impair the activity of the leptin receptor signaling pathway, we studied the effects of ADX and corticosterone replacement on components of the leptin receptor signaling pathway, including the hypothalamic leptin receptors, SOCS-3, STAT-3, and neuropeptide Y (NPY).

Methods

Animals. Ten-week-old male Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). They were housed individually in hanging wire mesh cages attached to an automated watering system in a room with a 12:12-h light-dark cycle (7:00 AM–7:00 PM) at a temperature of 22–23°C. All rats consumed nonpurified chow diet (Rodent chow 5001, Purina Mills, St. Louis, MO). Food was available ad libitum throughout the experiment. All protocols used were approved by the Pennington Center's Institutional Animal Care and Use Committee.

Intracerebroventricular cannulation. Rats weighing 270–300 g were anesthetized by intraperitoneal injection with 0.125 ml anesthetic per 100 g body wt [anesthesia mixture, ketamine (80 mg/ml), ace promazine (1.6 mg/ml), and xylazine (5 mg/ml)]. Rats were stereotactically implanted with a stainless-steel guide cannula (25 gauge, 14-mm long) into the third cerebral ventricle. The coordinates were 2.8 mm posterior to bregma, 0.0 mm lateral to midsagittal, and 8.1 mm ventral to the dura according to the brain atlas (46). Cannulas were secured in place with anchor screws and dental acrylic and occluded with 30-gauge wire stylet. The injector (31 gauge) projected 0.5 mm beyond the tip of the guide cannula. Body weights were monitored daily during the 7-day recovery period.

ADX. Seven days after recovery from cannula placement surgery, rats were bilaterally adrenalectomized via a dorsal approach under isoflurane anesthesia. One group of ADX rats was implanted subcutaneously with 3-wk release 50 mg corticosterone pellets (Innovative Research of America, Sarasota, FL). Sham operations followed similar procedures to ADX, but the adrenal glands were not removed. ADX rats were provided with 0.9% saline drinking water and allowed to recover for 7 days before leptin treatment. Body weights were monitored daily after ADX.

In vivo experiments. Intracerebroventricular microinfusions in unrestrained rats were at the rate of 1 μl/min using an infusion pump (Harvard Apparatus, South Natick, MA). Each animal was infused between 3:00 and 3:30 PM (3.5–4.0 h before dark onset). Rats received either vehicle (sterile physiological saline, 2 μl/rat) or 2.5 μg recombinant leptin (R&D Systems, Minneapolis, MN) per rat dissolved in 2 μl saline vehicle. Twenty-four-hour food intake and body weight were monitored. After 24 h, rats were given a second intracerebroventricular injection of saline or 2.5 μg leptin. Two hours later, the anesthetized rats were killed by decapitation, and trunk blood was collected for the determination of plasma hormones. The hypothalamus, epididymal fat pads, and liver were dissected from each rat, weighed, and snap-frozen in liquid nitrogen and stored at −80°C until used for total RNA and protein analyses. The dose of leptin and time of sampling chosen for these studies were based on our laboratory’s previous studies that determined the time and concentration dependence of leptin effects on food intake (36).

RNA analysis. Total RNA was extracted from whole hypothalamus as described previously (33) using TRIzol reagent (Life Technologies, Gibco BRL, Gaithersburg, MD). A ribonuclease protection assay for the leptin receptor was performed as described earlier (38). RT-PCR was used to determine mRNA expressions for SOCS-3 and STAT-3 using cyclophilin as an internal standard. Five micrograms of total RNA from individual hypothalamus was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) using oligo(dT)12–18. mRNA expression was determined by PCR using the following primer sequences: 5′-ACCAGCGCCACCTTCCTCACA-3′ and 5′-GTGGAGCAGATCACTGGTCC-3′ to amplify a 450-bp DNA fragment of SOCS-3 (GenBank AF075383), 5′-AAGGACATCGTGGC-3′ and 5′-ACACGGCCGACAGACAGATC-3′ to amplify a 715-bp DNA fragment of STAT-3 (GenBank X91810), and 5′-GACAAAATTCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAAG-3′ and 5′-ACT-TCAGTGGAGCAGAGATTACAGGG-3′ were used to amplify a 528-bp DNA fragment of cyclophilin (GenBank M19533). PCR products were then cloned directly into pCR2.1 (Invitrogen, Carlsbad, CA), and the identity of each cloned product was confirmed by sequencing using the ABI...
**Serum assays.** Plasma corticosterone, insulin, and leptin were measured with RIA kits (Linco Research, St. Charles, MO) based on rat standards according to the supplier’s instructions.

Statistics. Data are presented as means ± SE. Statistical analysis was performed using the Student’s t-test and two-way ANOVA. Post hoc analysis was performed using the Duncan’s multiple-range test method at $P < 0.05$.

**RESULTS**

The aim of this study was to examine the molecular basis for the increased sensitivity to leptin in ADX rats. Rats were sham-operated, adrenalectomized, and steroid-replaced with corticosterone pellets and adrenalectomized. Leptin or saline vehicle was administered intracerebroventricularly. Figure 1 shows the effects of ADX and leptin treatment on food intake. In saline-treated rats, ADX reduced 24-h food intake by 35% compared with sham-operated rats. Corticosterone treatment of saline-infused ADX rats returned the food intake to the level equivalent to that of sham-operated rats. Leptin treatment of sham-operated rats significantly decreased 24-h food intake by 43% compared with saline-treated rats. Treatment of ADX rats with leptin significantly decreased food intake by 73% compared with saline-treated ADX rats. Corticosterone treatment of ADX rats attenuated their response to leptin and restored the response to the level of that observed in the sham-treated rats.

ADX significantly reduced the epididymal fat pad weight compared with sham-operated rats (Table 1). Leptin treatment of ADX rats decreased epididymal fat pad weight by 32% compared with saline-treated ADX rats; however, this decrease was not significant. Corticosterone replacement in ADX rats attenuated lepin-induced fat pad weight loss and permitted weight gain to the level of saline-treated, corticosterone-replaced ADX rats. As with the slight body weight change, corticosterone-treated ADX rats had similar epididymal fat pad weights regardless of leptin treatment.

In sham-operated rats, leptin treatment reduced plasma insulin levels by almost 50% compared with saline treatment.
Table 1. Body weight, liver and epididymal WAT weights, and serum hormone levels of adrenalectomized, adrenalectomized and steroid-replaced, and sham-operated Sprague-Dawley rats

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<td>316.2±4.4</td>
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<td>0.63±0.29</td>
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<td>0.42±0.11</td>
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Values are means ± SE. Rats were injected with leptin (2.5 μg icv) or vehicle at 0 and 24 h and killed 2 h after the second injection. Significant difference between individual groups is indicated by the differences in letter symbols, with P < 0.05. There were 6–8 animals in each experimental group. ANOVA showed significant differences in body weight change (F(0.426, 6) = 6.16, P < 0.002), epididymal white adipose tissue (WAT; F(0.426, 6) = 3.84, P < 0.005), corticosterone (F(0.426, 6) = 21.17, P < 0.0001), leptin (F(0.426, 6) = 4.03, P < 0.01), but not insulin (F(0.426, 6) = 2.37, P = 0.07); ADX-R, adrenalectomized and steroid replaced; Sham, sham operated.

ADX significantly reduced plasma insulin levels compared with sham-operated rats. However, leptin treatment of ADX rats had no further effects on serum insulin. Corticosterone treatment of ADX rats showed a tendency to increase plasma insulin levels, but they did not reach the level of sham-operated rats. Corticosterone replacement of ADX rats increased serum corticosterone levels that were −50% of those seen in the sham-operated rats. Leptin treatment had no effect on serum corticosterone of the sham-operated group. Plasma leptin levels varied not only as a result of leptin infusion, but also in association with corticosterone depletion and replacement (Table 1). ADX significantly reduced the levels of plasma leptin. However, corticosterone did not elevate plasma leptin levels in saline-treated ADX rats. Plasma leptin was increased in rats infused with leptin. However, the increment in leptin levels in leptin-treated rats was far greater in sham-operated rats than in ADX rats, with the corticosterone-treated ADX rats showing intermediate levels.

ADX significantly increased both OBR-L and OBR-S mRNA levels compared with sham-operated rats, but corticosterone replacement of ADX rats failed to reverse these responses (Fig. 2). Leptin treatment had no effect on the levels of OBR-L and OBR-S mRNA in any experimental groups. The hypothalamic STAT-3 mRNA levels were increased after ADX (Fig. 3) compared with sham-operated rats. Glucocorticoid treatment of saline-infused ADX rats reduced the levels of STAT-3 mRNA. Leptin had no effect on STAT-3 mRNA expression in ADX or corticosterone-treated ADX rats compared with their saline-treated controls. However, leptin reduced STAT-3 mRNA expression in sham-operated rats compared with saline-treated rats.

Immunoprecipitation and Western blotting were used to show that ADX increased the levels of total STAT-3 protein levels compared with sham-operated rats and corticosterone treatment failed to reverse this effect (Fig. 4). Leptin treatment did not affect STAT-3 protein levels in any group. The discrepancy in the mRNA and protein responses to leptin in sham-operated rats may be due to differences in the temporal changes in mRNA and protein not being reflected in tissues sampled at a single time point.

We then investigated whether the ADX-induced increase in STAT-3 protein levels in hypothalamic lysates was associated with increased STAT-3 activity (Fig. 5). ADX induced STAT-3 protein tyrosine phosphorylation compared with sham-operated, saline-treated rats. Leptin treatment increased STAT-3 tyrosine phosphorylation in the hypothalamus of sham-operated and ADX rats compared with their saline-treated controls. Steroid replacement in ADX rats returned the levels of STAT-3 tyrosine phosphorylation to that of sham-operated, saline-treated rats, but leptin unexpectedly inhibited STAT-3 tyrosine phosphorylation in this group. To make sure that equal levels of protein were loaded on the gel and that efficiency of transfer was optimum, membranes were stripped and rebotted with a STAT-3 antibody. This verified that the tyrosine phosphorylated 92-kDa protein was STAT-3 and that each lane contained the same amounts of the STAT-3 protein. This, therefore, showed that both ADX and leptin treatment induced phosphorylation of STAT-3.

Nuclear extracts were also used to examine the effects of ADX and leptin treatment on STAT-3 DNA binding activity on the sis-inducible element from a c-fos promoter (m67-SIE) by using gel shift assays (Fig. 6). The DNA-protein complex was inhibited by an excess of unlabeled m67-SIE oligonucleotide (lane 3). Competition with STAT-3 antibody showed that the complex bound to the m67-SIE in response to ADX and leptin treatment was composed of STAT-3 (lanes 4 and 5). Leptin treatment of sham-operated rats enhanced the activation of STAT-3 compared with saline-treated rats. STAT-3 activation was enhanced in ADX and glucocorticoid-replaced ADX rats compared with sham-operated, saline-treated rats. The STAT-3 DNA binding activity of ADX rats was not further upregulated by leptin treatment, but rather decreased.

Recently, peripheral administration of leptin to ob/ob mice was shown to rapidly induce SOCS-3 mRNA in the hypothalamus, but this effect was absent in db/db mice lacking a functional OBR-L (3, 20). The
effects of ADX and leptin treatment on SOCS-3 mRNA expression (Fig. 7) and protein levels (Fig. 8) were examined by using semi-quantitative RT-PCR and Western blot analysis of immunoprecipitated SOCS-3, respectively. Leptin treatment had no effect on SOCS-3 mRNA expression, but increased SOCS-3 protein levels in all experimental groups. ADX decreased SOCS-3 mRNA expression and protein levels compared with sham-operated rats, and these effects were reversed by glucocorticoid treatment.

Activation of OBR-L by leptin treatment has recently been shown to induce tyrosine phosphorylation of the SH2-containing SHP-2 and its recruitment to the cytoplasmic domain of OBR-L (7). SHP-2 is thought to negatively regulate STAT-3-mediated gene induction after activation of OBR-L (7). Therefore, the effects of ADX and leptin treatment on the protein levels and phosphorylation of SHP-2 were examined (Fig. 9). ADX had no effect on the levels and phosphorylation of SHP-2 protein in any experimental group. However, leptin treatment appeared to increase both tyrosine phosphorylation of SHP-2 protein and the level of total SHP-2 protein, such that the proportion of protein that was tyrosine phosphorylated was unchanged.

NPY, a potent orexigenic peptide, is down- and up-regulated by leptin and glucocorticoids, respectively (47). The effects of ADX and leptin treatment on the expression of hypothalamic NPY mRNA were determined by using Northern blot analysis (Fig. 10). ADX significantly reduced NPY mRNA levels, and this effect was reversed by corticosterone administration. Leptin treatment of sham-operated and corticosterone-replaced ADX rats also significantly reduced NPY mRNA expression compared with saline-infused controls. However, no further decrease in NPY mRNA expression was observed in ADX rats treated with leptin.

DISCUSSION

The key findings of this study were 1) that the feeding response to leptin is sensitive to adrenal glucocorticoids, their absence enhancing the anorectic response to leptin; 2) that mRNA expression of the leptin receptor isoforms in the hypothalamus is increased by ADX; 3) that ADX increased STAT-3 mRNA expression and total protein levels; 4) that leptin treatment of sham-operated rats induced phosphorylation of STAT-3, and ADX induced constitutive phosphorylation of STAT-3 protein; 5) that ADX and leptin treatment induced STAT-3 DNA binding activity, but there was no synergy between ADX and leptin treatment; 6) that ADX decreased the expression of SOCS-3 mRNA and protein levels, whereas leptin treatment increased SOCS-3 protein levels; and 7) that the increased sensitivity to leptin on ADX is not due to further changes in NPY mRNA expression.

Our original observations that ADX increases the expression of both the long-form and short-form leptin

Fig. 2. Ribonuclease protection assay to determine the effect of ADX and leptin treatment on leptin receptor mRNA expression. Total RNA was extracted from individual hypothalami of rats treated intracerebroventricularly with either saline vehicle or 2.5 μg leptin. RNA was hybridized with 32P-labeled antisense probes for leptin receptor (ObR) and β-actin and processed as described in METHODS. A: representative ribonuclease protection assay for leptin receptor isoforms. B: histograms showing the quantification of the long-form and the short-form ObR (ObR-L and ObR-S, respectively) mRNAs. Open bars, saline-treated; filled bars, leptin-treated animals. M is century RNA molecular markers. Data are presented as means ± SE (n = 6–8 per group). *P < 0.05 compared with sham-operated rats.
receptors in the hypothalamus of obese Zucker rats suggested that adrenal glucocorticoids might modulate their effect on leptin response through modulation of leptin receptor activity (39). However, because ADX attenuates obesity in rodent models with mutations in the leptin receptor, it is unlikely that glucocorticoid-regulated changes on leptin receptors alone could explain the effects of ADX. Hence, we hypothesized that glucocorticoids not only modulate expression of the leptin receptor but also affect the leptin signal transduction pathway. Thus we investigated the effects of ADX on either gene expression, protein phosphorylation, or DNA binding activity of several components in the JAK-STAT signaling pathway, such as STAT-3, SOCS-3, SHP-2, and NPY. Our data are consistent with the published data (58) that showed that ADX rats have increased sensitivity to leptin treatment and suggest that ADX may prevent obesity through constitutive activation of the JAK-STAT signaling pathway. The ability to reverse obesity by activation of this pathway has previously been shown with the use of ciliary neurotrophic factor in db/db mice (26).

ADX caused an increase in the levels of mRNA for the long and short forms of the receptor. This was associated with a reduction in circulating leptin levels, which may suggest that the change in receptor expression may help to enhance the effects of leptin on food intake when leptin is administered directly into the brain. The data could also be interpreted as reflecting that leptin can modulate the expression of its own receptors, with receptor gene expression increasing when leptin levels decrease. However, if this is the case, it appears to be a delayed response in the absence of glucocorticoids, because acute treatment of ADX rats with leptin did not reduce the levels of either OBR-L or OBR-S mRNA. The increase in leptin receptor population in the hypothalamus may lead to increased receptor activity and thereby cause pronounced effects of leptin action. Caution is also required in the interpretation of mRNA levels for leptin receptor, because in previous studies we showed that receptor mRNA and protein levels do not necessarily change parallel to each other (38).

Leptin regulates food intake and body weight via interactions with hypothalamic neuronal circuits expressing leptin receptors (18). Binding to the long isoform of OBR initiates acute changes in neuropeptide release or longer-term changes in neuropeptide gene transcription. These effects may be mediated by a number of signaling pathways, including the JAK-STAT pathway of signal transduction. Recent evidence suggests that the STAT-3 transcription factor mediates some of leptin’s actions in the hypothalamus (2, 48, 57). We confirmed this in the present study, showing that leptin induced STAT-3 phosphorylation and DNA binding activity. ADX induced a similar but constitutive phosphorylation of STAT-3 and enhancement of DNA binding activity. This constitutive phosphorylation of STAT-3 was further increased by leptin treat-

![Fig. 3. Effects of ADX and leptin treatment on the hypothalamic signal transducers and activators of transcription-3 (STAT-3) mRNA expression. Total RNA was extracted from hypothalami of ADX, steroid-replaced ADX, and sham-operated rats. RNA was reverse transcribed and PCR amplified using STAT-3 specific primers. PCR products were fractionated in a 1.5% agarose gel for 2 h. A: representative agarose gel showing the 715-bp STAT-3 PCR product and cyclophilin as internal standard. B: histogram showing the quantification of the STAT-3 mRNA. Open bars, saline-treated; filled bars, leptin-treated groups. Data are presented as means ± SE for 6–8 observations per group obtained by running 2 individual gels. *P < 0.05, **P < 0.001, and ***P < 0.0001 compared with indicated groups.](http://ajpregu.physiology.org/)

![Fig. 4. Effects of ADX and leptin treatment on hypothalamic STAT-3 protein levels. Total STAT-3 protein levels were analyzed by immunoprecipitating STAT-3 from hypothalamic lysates of rats treated with either saline vehicle or 2.5 μg leptin using STAT-3 antibody followed by Western blotting and analysis with STAT-3 antibody. A: typical immunoblot of STAT-3. B: densitometric values of 3 immunoblots for 6 animals in each group. Open bars, saline-treated; filled bars, leptin-treated groups. Data are presented as means ± SE (n = 6 per group). *P < 0.05 compared with sham-operated rats.](http://ajpregu.physiology.org/)
ment. However, it was rather puzzling that the combined effects of ADX and leptin treatment on STAT-3 tyrosine phosphorylation did not translate into increased DNA binding. A similar observation was reported recently by Scarpace and colleagues (51), where leptin treatment of old rats increased the levels of phosphorylated STAT-3 but had no effect on STAT-3 DNA binding activity compared with young rats. These findings might be explained if the excess phosphorylated STAT-3 was bound by protein inhibitor of activated STAT-3 (PIAS3), as recently described (11). PIAS3 blocks the DNA binding activity of STAT-3, thus inhibiting STAT-3-mediated gene activation. We did not study other STAT proteins, especially STAT-1, because McCowen and colleagues (37) showed that leptin did not increase the phosphorylation of STAT-1 and STAT-5, despite abundant expression of these signaling molecules in the hypothalamus.

Cytokines transduce signals through the JAK-STAT pathway to increase the transcription of genes with STAT recognition sites in their promoters. SOCS proteins are a new family of negative regulators of cytokine signal transduction. The expression of SOCS proteins is induced by cytokines, including leptin (3). Once expressed, SOCS downregulate JAK-STAT pathways and hence modulate the biological response. A leptin-dependent increase of SOCS-3 mRNA has been reported in areas of the hypothalamus expressing high levels of OBR-L (3, 20). Furthermore, SOCS-3 was shown to block leptin-induced signal transduction in mammalian cell lines, suggesting that SOCS-3 is a leptin-inducible inhibitor of leptin signaling (3). In our study, leptin treatment of sham-operated rats increased SOCS-3 protein levels in the hypothalamus, consistent with other reports (3, 20). ADX decreased SOCS-3 mRNA expression and SOCS-3 protein levels. This decrease in SOCS-3 indicates dependence of SOCS-3 on the presence of glucocorticoids and provides a potential mechanism by which leptin resistance could be modulated by decreasing corticosterone levels. In support of this proposal for downregulation of SOCS-3 after ADX, a search for a putative glucocorticoid response element (GRE) in the rat SOCS-3 gene (GenBank AJ249240) identified a 393-agaaccaggca-403 base sequence. If indeed this is a GRE, then it will confirm the possibility that SOCS-3 can be upregulated by glucocorticoids. This upstream region also contains two putative STAT-3 binding elements that have been recently characterized (1) and shown to regulate SOCS-3 expression induced by leukemia inhibitory fac-

![Fig. 5](image1.png)

**Fig. 5.** Effects of ADX and leptin treatment on tyrosine phosphorylation of STAT-3 protein. Tyrosine phosphorylation of STAT-3 was analyzed by immunoprecipitating (IP) STAT-3 from hypothalamic nuclear extracts obtained from ADX, corticosterone-replaced ADX, and sham-operated rats treated with either saline vehicle or 2.5 μg leptin using STAT-3 antibody followed by Western blotting and analysis with antiphosphotyrosine (pY20) antibody. The membranes were stripped and reprobed with STAT-3 antibody. A: immunoblot of phosphotyrosine and STAT-3. B: densitometric values of the immunoblot data taken from 3 repeats of this experiment using samples from 3 individual rats in each experimental group. Open bars, saline-treated; filled bars, leptin-treated groups. Data are means ± SE (n = 3 per group). *P < 0.05 compared with indicated groups. IB, immunoblot; OD, optical density.

![Fig. 6](image2.png)

**Fig. 6.** Effects of ADX and leptin treatment on the DNA binding activity of STAT-3 protein. Nuclear extracts were incubated with 32P-labeled c-fos nis-inducible element (m67-SIE) and fractionated on a 5% nondenaturing PAGE. A: representative electrophoretic mobility shift assay (EMSA) gel. B: densitometric quantification of the STAT-3-DNA complex. The EMSA was repeated 3 times so that 6 animals from each group were assayed. Open bars, saline-treated; filled bars, leptin-treated groups. Lane 1, DNA only; lane 2, DNA and unrelated DNA; lane 3, competition with 100-fold molar excess of cold m67-SIE; lanes 4 and 5, complex in the presence of 1 and 3 μL STAT-3 antibody. Data are presented as means ± SE (n = 6 per group). *P < 0.05 and **P < 0.005 compared with indicated groups.
tor (LIF). Our studies showed that, in the absence of glucocorticoids, leptin activation of STAT-3 led to a small increase in SOCS-3 protein levels, whereas sham-operated rats showed a much greater stimulation of SOCS-3 after leptin treatment. This could suggest that STAT-3 activation and glucocorticoids act in a concerted manner to regulate SOCS-3 expression. Furthermore, it provides a mechanism for the enhanced response to leptin that is observed after ADX.

It is not possible in vivo experiments to confirm that glucocorticoids directly regulate gene transcription of OBR, STAT-3, or SOCS-3. Clearly, changes in glucocorticoid levels result in alterations in the secretion of other hormones (e.g., insulin) and cytokines [e.g., interleukin (IL)-6] that could be responsible for the observed changes in mRNA levels of these genes in ADX rats.

The induction of SOCS genes, by STAT elements present in the SOCS promoter, has so far been restricted to activators of the cytokine receptor family, such as leukemia inhibitory factor, IL-6, interferon-γ, growth hormone, and leptin. Although STAT activation is the hallmark of cytokine action, it can be activated by other agents, such as ANG II, epidermal growth factor, and platelet-derived growth factor. Recent evidence, therefore, indicates that STAT proteins can be activated by a variety of receptor and nonreceptor protein-tyrosine kinases (30). Unlike cytokine-induced activation of STATs, where JAKs are known to play a pivotal role in phosphorylating STATs, the mechanism for receptor protein-tyrosine kinase-mediated activation of STATs remains elusive. Insulin modulates cellular metabolism by modifying the activity and intracellular localization of several proteins and by modulating transcription (43). Recently, insulin has been shown to induce tyrosine phosphorylation and DNA binding activity of STAT-5B in a perfused rat liver (10), showing its involvement in the regulation of transcription by STAT factors. A role for STAT-3 in insulin regulation of SOCS-3 mRNA has not been ruled out (19). ADX and leptin reduced insulin levels, but nevertheless STAT-3 activation was increased in the hypothalamus of ADX rats. Thus it seems unlikely that the fall in insulin could explain the reduction in SOCS-3 mRNA and protein after ADX.

Chavez and colleagues (8) showed that ADX increased sensitivity to central insulin administration, similar to the increase in leptin sensitivity after ADX reported in this manuscript and previously described by Zakrzewska and colleagues (58). Whether the increased insulin sensitivity of ADX rats plays a role in the changes in SOCS-3 levels and in the STAT activation observed in the ADX rats remains to be investi-
However, these two studies, together with the current data, suggest that the absence of glucocorticoids increases the brain’s sensitivity to both leptin and insulin and that enhanced actions of these hormones to lower food intake and body weight may be an important component of the ability to prevent the development of obesity by removal of adrenal glucocorticoids.

Previous reports have indicated that central administration of leptin inhibits insulin secretion and increases insulin sensitivity of peripheral tissues directly (13, 28, 33, 42). In the current studies, this leptin effect did not quite achieve statistical significance in sham-treated rats but was clearly absent in ADX and steroid-replaced rats. This could be interpreted to suggest that leptin effects on insulin secretion may require circulating glucocorticoids. Alternatively, the central effects of leptin on insulin secretion are likely mediated through changes in autonomic activity. ADX, in enhancing sympathetic activity and decreasing parasympathetic drive, has identical effects to leptin. Leptin also has direct effects on pancreatic β-cells to inhibit insulin secretion via its action on the receptor and by activation of ATP-sensitive K⁺ channels (34, 45, 52). It is possible that these leptin inhibitory effects on insulin secretion and insulin gene expression in pancreatic β-cells may also be modulated by glucocorticoids.

Leptin modulates its effect on food intake and energy expenditure through its effects on multiple neuropeptide systems, including NPY and proopiomelanocortin/α-melanocyte stimulating hormone (POMC/α-MSH; 5, 17, 21, 56). Both ADX and leptin administration decrease NPY gene expression and release (9, 32). However, in the study reported here, leptin had no additional suppressive effect on the reduced level of NPY mRNA observed in the ADX rats. This confirms recent observations (54), which also showed that ADX-induced sensitivity to leptin was not due to a further decrease in NPY. This suggests that the enhanced response to leptin in ADX rats is more likely associated with enhanced expression of the genes for anorectic peptides (e.g., POMC; Ref. 40) rather than additional
suppression of orexigenic peptides. Jang and colleagues (32) showed that leptin increases CRH secretion in ADX rats, which could also mediate the increased sensitivity to leptin.

Although the observations reported in this study provide a mechanistic explanation for the effects of glucocorticoids in promoting obesity, it is important to recognize that our interpretation is based on changes in whole hypothalami. Specific neuronal populations within the medial and lateral hypothalamus are responsive to leptin. Thus it will be necessary to confirm, using in situ hybridization and immunohistochemistry, that the changes in the STAT and SOCS systems that we have reported are reflective of changes in the specific neuronal pathways that are known to be influenced by leptin.

Perspectives

We have shown that ADX increases sensitivity to exogenous leptin, enhances leptin receptor mRNA expression, induces a constitutive STAT-3 activation, and reduces the expression of the cytokine inhibitor SOCS-3. These studies provide an understanding of the mechanism through which adrenal glucocorticoids modulate the leptin signaling pathway and influence the hypothalamic systems that control energy balance.

The association of hyperleptinemia with obesity has led to the concept of leptin resistance. Such resistance may be modulated at multiple steps from transport into the central nervous system to changes in the target genes (49). Our data suggest that adrenal glucocorticoids may either directly or indirectly modulate those responses to leptin that are mediated through the JAK-STAT pathway (51). Aging also appears to be associated with a reduction in activity of this JAK-STAT pathway. The attenuated response of elderly rats to dietary signals and their tendency to gain weight may also be prevented by ADX (49). This suggests that glucocorticoid activity might also be a contributor to the impairment in JAK-STAT signaling observed in elderly rats and further supports the concept that activation of the postreceptor signaling pathways for leptin would be an excellent target for pharmacotherapy of obesity.

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