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Regulation of Leptin Expression and Secretion by Corticosteroids and Insulin

Implications for Body Weight

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Leptin is an important hormone that has potent effects on appetite and body weight. The regulation of leptin gene expression and secretion by corticosteroids and insulin was studied in the rat. Adrenalectomy resulted in a significant reduction in leptin gene expression and secretion. The reduction was corrected by hormonal replacement with corticosterone pellets, showing that normal levels of circulating corticosteroids are required to maintain leptin expression and secretion in the body. Chronic treatment with dexamethasone (DEX) over 3 wk did not significantly increase leptin gene expression and secretion, contrary to earlier reports using shorter treatment paradigms. The profound weight loss associated with chronic DEX treatment may have abrogated the direct stimulatory effect of DEX on leptin gene expression and secretion, indicating a possible crosstalk between corticosteroids and leptin in the regulation of body weight. Shorter-term treatment of animals with DEX (3.7 μg/g body wt; 24 h) increased leptin gene expression and secretion about 2-fold and 1.4-fold, respectively. The increase was independent of circulating insulin concentrations. In streptozotocin-treated rats, short-term DEX treatment increased leptin gene expression and secretion about 3.5-fold and 2-fold, respectively. The data indicate that circulating leptin concentrations and adipose tissue leptin expression are dependent on corticosteroids and insulin. Although acute DEX treatment resulted in a stimulatory effect on leptin secretion and expression, chronic DEX treatment did not. The stimulatory effect of DEX on leptin is independent of circulating insulin concentrations

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Introduction

Energy balance is tightly regulated in the body by a variety of different hormones, including glucocorticoids and insulin *(1)*. Recently, the mouse obese *(ob)* gene and its human homolog was identified and cloned using positional cloning techniques *(2)*. The *ob* gene is specifically expressed in adipocytes and encodes a 167 amino acid secreted peptide called leptin *(2–6)*. It has been postulated that leptin acts as a feedback signal to regulate energy stores in the body *(7,8)*. In support of this hypothesis, plasma leptin concentrations and mRNA levels in adipose tissue change in parallel with body weights and fat stores *(4,9,10)*. Leptin is thought to act on the hypothalamus to effect changes in appetite and metabolism to control energy balance *(7,8,11)*.

The potent effects of leptin on energy balance in the body has prompted studies on the regulation of leptin gene expression and secretion. Both corticosteroids *(5,12–14)* and insulin *(15–17)* have been shown to have effects on leptin gene expression and secretion. Recently, De Vos et al. *(12)* showed that catabolic doses of glucocorticoids resulted in a decrease in body weight gain and food intake, which were correlated to an increase in leptin mRNA expression in epididymal fat. The glucocorticoid-induced decrease in body weight gain and food intake was thought to be mediated by changes in leptin gene expression. Although short-term treatment with glucocorticoids has the ability to enhance leptin expression and secretion, the longer term physiological effects have not yet been fully characterized. In this present work, we studied the effects of adrenalectomy and corticosterone replacement, as well as dexamethasone (DEX) treatment on leptin mRNA and plasma leptin concentrations. Since glucocorticoids are known to increase plasma insulin levels *(1)*, we also studied the involvement of insulin in mediating the stimulatory effect of DEX on leptin gene expression and secretion in the body.

Results

Effects of Adrenalectomy and DEX on Plasma Leptin and Leptin mRNA Levels

Bilateral adrenalectomy resulted in a significant decrease in weight gain. Adrenalectomized rats gained only about 50 g over the 3 wk compared to more than 100 g in the controls (Table 1). Implantation of DEX pellets, however, resulted in a dramatic weight loss of more than 70 g over the 3 wk. The efficacy of the various treatments was verified by the plasma corticosterone concentrations in the animals. Adrenalectomy resulted in a precipitous decline in plasma corticosterone concentrations 1 and 3 wk after adrenalectomy to undetectable levels. Similarly, long-term exposure to DEX also reduced plasma corticosterone concentrations after 3 wk to levels below the sensitivity of the corticosterone assay, showing a powerful suppression of endogenous secretion of corticosterone.

The rat leptin probe hybridized to a specific band in the rat epididymal fat Northern blot, which was estimated to be about 4.5 kb in size. The size corresponds to the mouse and human mRNA encoding leptin *(2,18)*. Adrenalectomy decreased leptin gene expression by about 60% in epididymal fat (Fig. 1A,B). Interestingly, the decrease in leptin expression was already maximal 1 wk after adrenalectomy and remained depressed for the entire 3 wk experimental period (ADX1 and ADX3). Continuous treatment of the animals with slow-release DEX pellets (25 mg) over 3 wk resulted in a 30% increase in leptin gene expression, which was not statistically significant.

Similarly, adrenalectomy resulted in a significant 45 and 83% decrease in plasma leptin concentrations 1 wk and 3 wk after adrenalectomy, respectively (Fig. 1C). Chronic DEX treatment over 3 wk at the dose used did not significantly increase plasma leptin concentrations over control values.

Effect of Corticosterone Replacement on Plasma Leptin and Leptin mRNA Concentrations

In order to determine if the effect of adrenalectomy on plasma leptin and leptin mRNA levels was owing specifically to the absence of corticosterone, we studied the effect of hormonal replacement of adrenalectomized animals with corticosterone. The effects of the various treatments on body weight gain and plasma corticosterone are shown in Table 2. Both control and adrenalectomized animals gained a similar amount of weight over the 1-wk period. On the other hand, corticosterone treatment of both adrenalectomized and sham-operated rats resulted in a significant loss of weight. The adrenalectomized rats lost about 10 g and the corticosterone treated sham-operated animals lost about 13 g over the 1-wk period. Similar to the first experiment, adrenalectomy resulted in a significant 40% decrease in leptin gene expression after 1 wk (Fig. 2A). Replacement of the animals with corticosterone for 1 wk restored leptin gene expression to control levels. Corticosterone treatment did not significantly increase leptin gene expression above control levels.

Table 1
Effect of Adrenalectomy and DEX on Body Weights and Plasma Corticosterone[a]

Groups	Weight gain, g	Plasma corticosterone, ng/mL
CTR	101.70 ± 8.32	79.38 ± 1.047
ADX1	31.80 ± 7.39[b]	Nondetectable
ADX3	49.88 ± 8.87[b]	Nondetectable
DEX	–72.47 ± 3.64[b]	Nondetectable

[a]The animals were weighed on the day of surgery and just before decapitation. Difference in weight was calculated for each animal. Results shown are means ± SEM.

[b]Indicates $p < 0.05$ relative to CTR. CTR—sham controls sacrificed after 3 wk; ADX1 and ADX3-bilateral ADX sacrificed after 1 wk and 3 wk, respectively; DEX-sham with DEX treatment for 3 wk.

Plasma leptin concentrations paralleled changes in leptin mRNA levels (Fig. 2B). Similar to the first experiment, adrenalectomy resulted in a 40% decrease in plasma leptin concentrations after 1 wk. This was restored to control levels with corticosterone replacement for 1 wk. Corticosterone treatment alone did not significantly alter plasma leptin concentrations compared to controls.

Effect of Streptozotocin (STZ) and DEX on Plasma Leptin and Leptin mRNA Concentrations

Although chronic DEX treatment did not significantly affect leptin mRNA and plasma leptin concentrations in our study (experiment 1), there are reports showing that DEX treatment increased leptin gene expression in the short term *(5,12)*. We studied the effect of short-term DEX treatment on leptin mRNA and plasma leptin concentrations and the influence of insulin on the DEX effect.

Changes in body weights, blood glucose concentrations, plasma corticosterone, and insulin concentrations resulting from the treatments are shown in Table 3. The efficacy of the STZ treatment is evident from the blood glucose and insulin concentrations. STZ treatment significantly increased blood glucose concentrations to about 400 mg/100 mL and decreased plasma insulin concentrations by about 75%. Acute DEX treatment reduced plasma corticosterone to undetectable levels, showing potent suppression of the adrenal axis. DEX significantly increased plasma insulin concentrations in non-STZ-treated animals by about 50% ($p < 0.05$). However, DEX treatment in STZ-treated animals did not significantly increase plasma insulin levels compared to STZ controls.

STZ treatment decreased leptin mRNA levels by about 70% (Fig. 3A,B). A single subcutaneous dose of DEX (3.7 μg/g body wt) increased leptin gene expression by 1.9-fold in non-STZ-treated animals. In STZ-treated animals, a single DEX injection increased leptin gene by about 3.5-

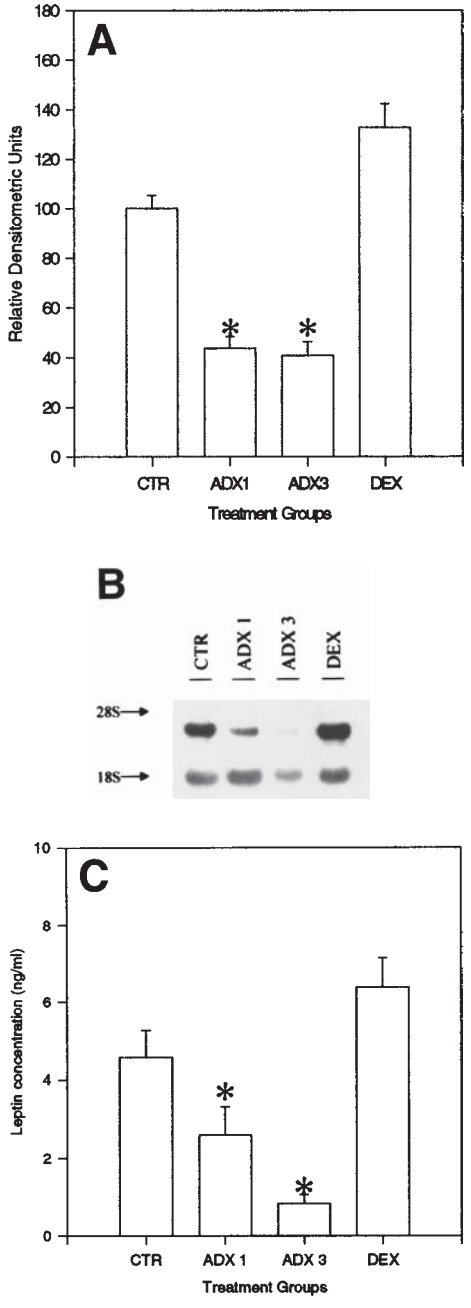

Fig. 1. Effect of adrenalectomy and DEX on leptin mRNA levels (**A** and **B**) and plasma leptin concentrations (**C**). Male Sprague Dawley rats were divided into the four groups as described in Materials and Methods. (B) shows a representative Northern blot of leptin and 18S RNA hybridization. Results in A and C are means ± SEM. * indicates $p < 0.05$, relative to CTR. CTR—sham controls sacrificed after 3 wk; ADX1 and ADX3—bilateral adrenalectomy, sacrificed after 1 wk and 3 wk, respectively; DEX—sham with DEX treatment for 3 wk.

fold compared to the STZ-treated group. Similarly, plasma leptin concentrations were decreased about 60% after STZ treatment (Fig. 3C). A single injection of DEX increased plasma leptin concentrations 1.4-fold compared to controls. In STZ-treated animals, DEX treatment increased plasma leptin concentrations by about twofold.

Table 2
Effect of Adrenalectomy and Corticosterone on Body Weights and Plasma Corticosterone[a]

Groups	Weight gain, g	Plasma corticosterone, ng/mL
CTR	54.28 ± 1.81	85.00 ± 39.865
ADX	46.24 ± 6.41	2.34 ± 0.73[b]
ADX/C	–9.65 ± 5.01[b]	146.13 ± 15.44
CORT 1	–12.80 ± 6.94[b]	144.24 ± 17.96

[a]CTR—sham controls; ADX—bilateral ADX; ADX/C—bilateral ADX with corticosterone replacement; CORT 1—sham with corticosterone treatment. The animals were sacrificed after 1 wk. Results shown are means ± SEM.
[b]Indicates $p < 0.05$, relative to CTR.

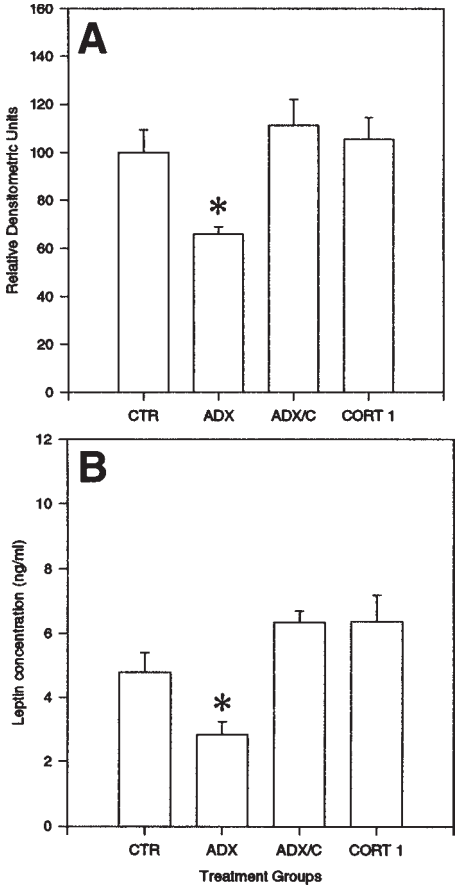

Fig. 2. Effect of adrenalectomy and corticosterone on leptin mRNA levels **(A)** and plasma leptin concentrations **(B)**. Male Sprague Dawley rats were randomly divided into four groups as described in Materials and Methods. CTR–sham controls; ADX—bilateral adrenalectomy; ADX/C–bilateral adrenalectomy with corticosterone replacement; CORT 1–sham with corticosterone treatment. The animals were sacrificed after 1 wk. Results shown are means ± SEM. *Indicates $p < 0.05$, relative to CTR.

Discussion

Energy balance is tightly regulated in the body. Both short-term and long-term signals are involved in orchestrating the tight regulation of energy balance and fat stores

Table 3
Effect of STZ and DEX on Blood Glucose, Plasma Corticosterone, and Insulin Concentrations[a]

Groups	Blood glucose, mg/100 mL	Plasma corticosterone, ng/mL	Plasma insulin, uiu/mL
CTR	10.2 ± 5.62	89.11 ± 1.42	30.78 ± 2.63
STZ	403.8 ± 17.98[b]	83.17 ± 21.38	8.31 ± 0.78[b]
DEX	106.0 ± 8.51	Nondetectable	45.95 ± 5.98[b]
STZ/D	396.6 ± 17.98[b]	Nondetectable	9.07 ± 1.40[b]

[a]CTR—saline-injected controls; STZ—streptozotocin-injected (125 mg/kg body wt); DEX—DEX-injected (3.7 μg/g body wt); STZ/D—streptozotocin- and DEX-injected (125 mg/kg body weight and 3.7 μg/g body wt, respectively). Results shown are means ± SEM.
[b]Indicates $p < 0.05$, relative to CTR.

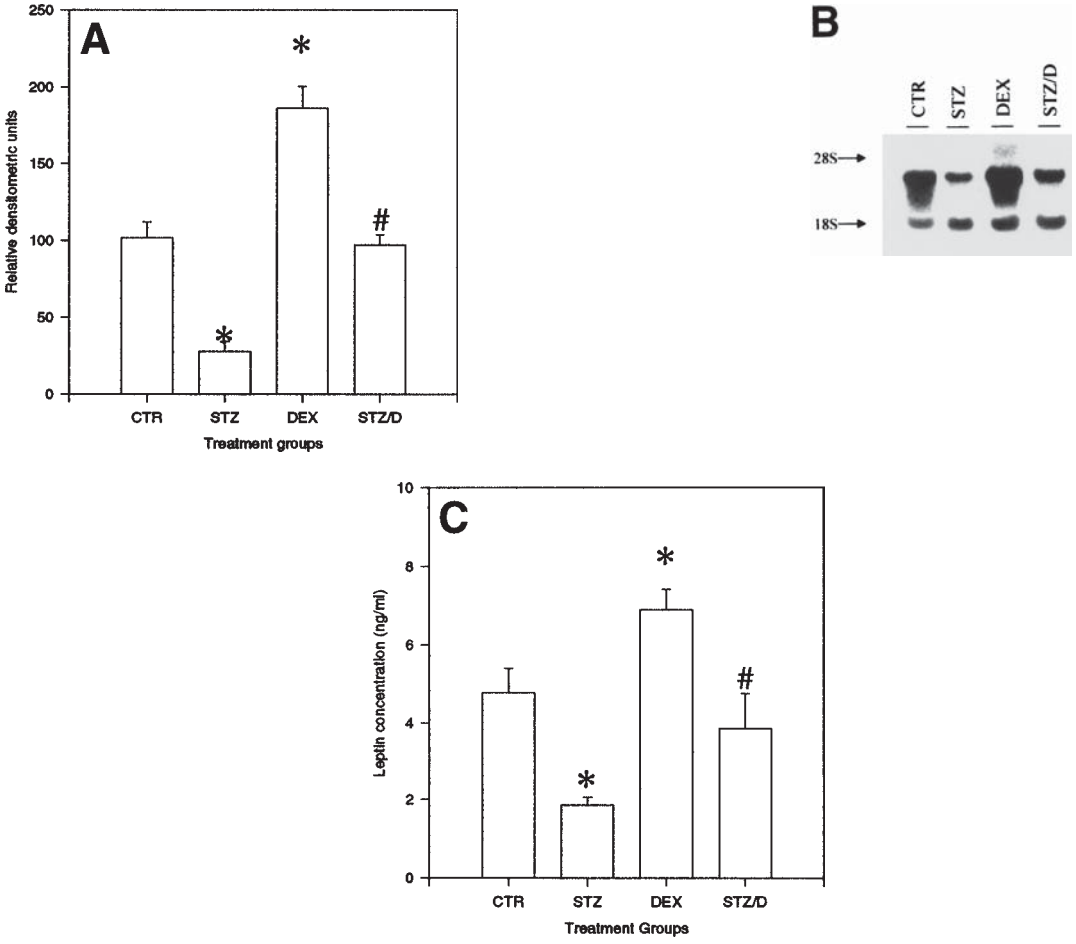

Fig. 3. Effect of STZ and DEX on leptin mRNA levels (**A** and **B**) and plasma leptin concentrations **(C)**. Male Sprague Dawley rats were randomly divided into four groups as described in Materials and Methods. (B) shows a representative Northern blot of leptin and 18S RNA hybridization. CTR—saline-injected controls; STZ—streptozotocin-injected (125 mg/kg body weight); DEX—dexamethasone-injected (3.7 μg/g body wt); STZ/D—streptozotocin- and DEX-injected (125 mg/kg body weight and 3.7 μg/g body wt, respectively). Results shown are means ± SEM. *Indicates $p < 0.05$, relative to CTR. #Indicates $p < 0.05$, relative to STZ.

(1,19). Leptin, the recently identified gene product of the *ob* gene *(2)* is a potent and important regulator of energy balance in the body. It is thought to act as a feedback hormone that signals the amount of fat stores in the body to the hypothalamus. The hypothalamus in turn effects changes in appetite and metabolism to regulate energy stores. Corticosteroids are also important long-term hormonal signals that regulate energy balance *(20)*. Corticosteroids act centrally to increase food intake and decrease metabolism *(20,21)*. In contrast, leptin acts centrally to decrease food intake and increase metabolism *(6–8,22)*. The integration of the two hormonal signals by central mechanisms to control energy balance is well illustrated in *ob/ob* mice lacking functional leptin. The development of the obese phenotype

in these mice is dependent on the presence of corticosteroids. Adrenalectomy resulted in a dramatic weight loss in the obese mice *(21)*, less in lean mice *(23–25)*. Intracerebroventricular injections of DEX in adrenalectomized *ob/ob* mice increased food intake and depressed metabolic rates, restoring body weights in these mice. Interestingly, injections of DEX in lean mice did not significantly affect food intake and oxygen consumption (12). It is possible that the presence of functional leptin in these lean mice abrogated the central DEX effects.

Similarly, corticosteroids are thought to act as counterregulatory hormones of leptin. Intracerebroventricular injections of leptin into nonadrenalectomized rats were not as efficacious in reducing body weight and food intake compared to adrenalectomized rats *(26)*. The replacement of adrenalectomized rats with DEX significantly decreased efficacy of leptin, showing an interaction between leptin and corticosteroids in the regulation of body weight and appetite in the body.

The potential interaction between the corticosteroids and leptin in the regulation of energy balance is also evident in the periphery. DEX increases leptin gene expression both in vivo and in vitro *(5,12,13,27)*. The results of the first and second experiments in this study suggests that corticosteroids are necessary to maintain normal expression and plasma concentrations of leptin in the body. The complete removal of corticosteroids in bilateral adrenalectomized animals resulted in a significant decrease in the basal expression and secretion of leptin in the body. Hormonal replacement of adrenalectomized animals with corticosterone restored normal concentrations of leptin gene expression and secretion. The normalization of leptin levels in the blood and adipose tissue occurred in the presence of weight loss in the ADX/C replaced animals, showing a specific effect of glucocorticoids on leptin. The 200-mg 21-d release corticosterone pellets used as replacement resulted in a release rate of 400 μg/h. Previous studies have shown that a rate of 200 μg/h corticosterone was sufficient for replacement after ADX *(28)*. However, 400 μg/h was recommended by the manufacturer to allow replacement in the higher physiological range. The decrease in plasma leptin gene concentrations and leptin mRNA levels in adipose tissue 3 wk after adrenalectomy was associated with a decrease in body fat stores observed at sacrifice and a significant decrease in weight gain compared to sham-operated controls (Table 1 CTR vs ADX3). It is likely that the reduction in plasma leptin concentrations in adrenalectomized rats abrogates in part the severity of weight loss that would otherwise occur. In *ob/ob* mice, which do not have functional leptin, the weight loss after bilateral adrenalectomy is severe relative to their original weight *(21)*. In the second experiment, there was a reduction in weight gain at 1 wk, although it was not significant compared to controls, potentially owing to the presence of low, but detectable levels of corticosterone in ADX animals compared with undetectable levels in experiment 1. It is likely that the reduction in plasma leptin concentrations and leptin mRNA at 1 wk abrogated the reduction in weight consequent to adrenalectomy.

Supraphysiological doses of DEX caused a suppression in the hypothalamic–pituitary–adrenal axis and induced a significant reduction in weight after 3 wk. DEX treatment for 3 wk resulted in modest, but not significant, increases in plasma leptin concentrations and leptin mRNA levels. The nonsignificant increase in leptin expression and secretion with DEX treatment reported in this study is in contrast to findings reported by others. De Vos et al. *(12)* reported a fivefold increase in leptin gene expression with DEX treatment for 4 d. A significant difference between the two studies is the length of treatment. It is likely that the long treatment employed in this study resulted in a more modest stimulation of gene expression compared to other shorter-period treatment paradigms. Although corticosteroids have anabolic effects centrally, they have catabolic effects in the periphery. The longer treatment resulted in a noticeable decrease in fat stores and a highly significant decrease in body weight. The decrease in body weight and fat stores would have resulted in a feedback inhibition of leptin gene expression, and this may have abrogated in part the direct stimulatory effect of DEX on leptin gene expression. It is also notable that the fivefold increase in leptin gene expression reported by De Vos et al. was associated with a <10% reduction in body weight with DEX treatment. The body weight of the DEX animals in our study were about 50% lower than the controls. The controls gained about 44% of their original body weight after 3 wk. In contrast, over the same period the DEX animals lost about 29% of their original body weight. It is also possible that higher catabolic doses of glucocorticoids are necessary for the leptin response. The 5 mg DEX pellet used in our study results in a daily dose of about 240 μg/animal. Given that the animals were about 250 g each, the dose used was approx 1 μg/g body wt, which was 3.7 times less than the dose used in the De Vos study *(12)*. In addition, the pattern of administration of the glucocorticoid in this study was continuous exposure using pellet implants in contrast to the De Vos study *(12)* using injections. This may also account for the differences in leptin responses observed. Similarly, in experiment 2, treatment of animals with corticosterone pellets for 1 wk did not significantly alter leptin expression and secretion. Although the plasma corticosterone levels in the corticosterone-treated animals were higher compared to controls, it was not significantly increased. However, the increase in plasma corticosterone in the animals was sufficient to cause significant weight loss (Table 2). It is possible that the combined effects of an insignificant increase in plasma corticosterone levels and a significant weight loss resulted in the lack of an effect of corticosterone treatment on leptin expression and secretion in the animals.

In addition to its effects on food intake and metabolic rates, corticosteroids also increase plasma insulin concentrations through an unknown mechanism *(1)*. Consistent

with this, DEX treatment of the animals resulted in a significant increase in plasma insulin concentrations. In addition, the third experiment in this study also demonstrated that basal levels of circulating insulin are necessary to maintain normal leptin gene expression and plasma leptin concentrations in the body. In the STZ-treated rats, insulin concentrations were severely decreased (Table 3). This results from the toxic effects of STZ on the pancreas. Under these circumstances, plasma leptin concentrations and leptin mRNA levels were also significantly decreased by about 60 and 70%, respectively after 2 d. In contrast to the lack of response to DEX in the first experiment, a single SC injection of DEX (3.7 μg/g body w) in the third experiment resulted in a significant 1.4-fold increase in plasma leptin concentrations and a twofold increase in leptin mRNA levels in non-STZ animals. The dose of DEX used in experiment 3 was the same as that used by De Vos et al. *(12)*. However, the animals were sacrificed 24 h after the DEX injection in our study, in contrast to 4 d in the DeVos study. This may have accounted for the lower response (twofold) of leptin mRNA levels compared to the fivefold observed in the De Vos study. Similarly, DEX also significantly increased both plasma leptin concentrations and leptin mRNA levels in STZ-treated animals (relative to STZ-treated alone). Interestingly, the DEX effect in STZ-treated animals was higher compared to that in non-STZ-treated animals. The results are consistent with a recent report showing an unexpected inhibition of DEX-stimulated leptin release in human adipocytes by insulin *(29)*. The results also suggest that insulin is not required for DEX induction of leptin gene expression. This is consistent with earlier reports showing a direct stimulatory effect of DEX on leptin gene expression in adipose tissue. The direct effect of DEX on leptin gene expression may be the mechanism by which it exerts a catabolic effect in the periphery. Stimulation of leptin gene expression and secretion results in higher plasma leptin concentrations that act on the hypothalamus to decrease appetite and increase metabolism resulting in weight loss.

In conclusion, the stimulatory effects of corticosteroids are necessary to maintain normal concentrations of circulating leptin and expression in adipose tissue. Although corticosteroids *(5,12–14)* and insulin *(15–17)* have been shown to have stimulatory effects on leptin, the studies in this paper show that both circulating concentrations of leptin and normal leptin expression in adipose tissue are dependent on circulating corticosteroids and insulin, showing physiological regulation of leptin in the body. The demonstration of physiological regulation of leptin by corticosteroids and insulin is important in view of conflicting reports on their effects on leptin *(30,31)*. In addition, we show that DEX stimulates leptin gene expression and secretion in adipose tissue independent of insulin. The regulation of leptin by corticosteroids when examined in the context of body weight changes indicate that there is likely crosstalk between the hypothalamic–pituitary–adrenal axis and leptin in the regulation of body weight.

MATERIALS AND METHODS

Animals and Treatments

Male Sprague Dawley rats (200–250 g) were used. Prior to surgery, the animals were housed in group cages with a maximum of four animals/cage. After surgery, they were housed individually. The cages were placed in an animal room on a 12:12 h light/dark cycle (lights on, 0700–1900 h). The animals were given free access to water and standard rat chow (Glen Forrest Stock Feeder, Australia). All surgeries were performed under sodium pentobarbital anesthesia (Nembutal, Boehringer Ingelheim, Australia).

Experiment 1: Effect of Adrenalectomy and DEX on Plasma Leptin and Leptin mRNA Levels

The animals were either sham-operated ($n = 10$) or bilaterally adrenalectomized ($n = 12$). A subset of the sham-operated animals ($n = 5$) was implanted with SC DEX pellets (5 mg, 21-d release; Innovative Research of America, Sarasota, FL). The remaining five sham-operated animals served as controls (CTR). The bilaterally adrenalectomized rats were supplemented with 0.9% saline drinking water for either 1 wk (ADX1) or 3 wk (ADX3). The animals were weighed on the day of surgery and just before sacrifice. All the animals were decapitated 3 wk after surgery, except for the ADX1 group, which were killed at the end of 1 wk.

Experiment 2: Effect of Adrenalectomy and Corticosterone on Plasma Leptin and Leptin mRNA Levels

The animals were randomly divided into four groups ($n = 5$ in each group). All the animals were either sham-operated or bilaterally adrenalectomized. Of the sham-operated animals, a group ($n = 5$) received SC 21-d slow release corticosterone pellets (CORT; 200 mg; Innovative Research of America). The other sham-operated group served as controls (CTR). One group of bilaterally adrenalectomized rats received SC 7-d slow-release corticosterone pellets as hormonal replacement (ADX/C). All the animals were killed by decapitation after 1 wk.

Experiment 3: The Role of Insulin in DEX-Induced Increase in Plasma Leptin and Leptin mRNA Levels

To determine whether the effect of DEX on leptin secretion and mRNA levels is dependent on the induction of plasma insulin, the effect of DEX treatment on controls and STZ-treated rats was studied. The animals received a single SC injection of either saline vehicle ($n = 10$) or STZ (Sigma Chemical, St. Louis, MO, 125 mg/kg body weight; $n = 10$) at 0900 h on d 1. Blood glucose concentrations were determined at 0900 on d 2 using an Accutrend glucometer (Boehringer Mannheim, Indianapolis, IN). The saline-injected rats were given either SC DEX injections (DEX; $n = 5$; 3.7 μg/g body wt, Dexasone, Atlantic Laboratories Corp., Thailand) or saline vehicle (CTR; $n = 5$) at 0900 on d 2. Similarly, the STZ-treated rats received either DEX (STZ/D) or saline vehicle injections (STZ) at 0900 on d 2. The animals were killed by decapitation at 0900 on d 3.

Collection of Tissues and Blood

At sacrifice, trunk blood was collected in prechilled tubes containing 400 µL aprotinin/10% tetrasodium EDTA (1:1). Epididymal fat was dissected and immediately frozen on dry ice. Samples were stored in a –80°C freezer until use. Plasma was obtained by centrifugation and aliquots stored at –80°C for subsequent determination of plasma corticosterone, insulin, and leptin concentrations.

Radioimmunoassays (RIA)

The plasma corticosterone was determined using a rat corticosterone RIA (ICN Biomedicals, Costa Mesa, CA). One hundred microliters of a 1:50 dilution or neat plasma were used. Plasma was diluted with assay buffer provided by the manufacturer. The assay does not crossreact with DEX. The intra-assay coefficient of variation for the assay was 8.25%. All the samples were assayed in a single assay. The plasma insulin concentration was determined using a human insulin RIA, which crossreacts 100% with rat insulin (ICN Biomedicals). The assay procedure was as described by the supplier. One hundred microliters of plasma was used for the assay. The intra-assay coefficient of variation for the assay was 7.8%. Plasma leptin was measured using a specific rat leptin RIA (Linco Research, St. Charles, MO). One hundred microliters of plasma were used for the assay. The intra-assay coefficient of variation was 7.6%.

Total RNA Extraction and Northern Blot Analysis

Total RNA was prepared using the guanidinium-thiocyanate method as previously described *(32)*. RNA was separated by formaldehyde/agarose gel electrophoresis, transferred to nylon membrane (Qiabrane, Qiagen Inc., Chatsworth, CA), crosslinked (Stratalinker, Stratagene, La Jolla, CA), and hybridized with complementary DNA (cDNA) probes.

The rat leptin probe (pOb22) was provided by L. M. Kaplan (Massachusetts General Hospital, Boston, MA). The pOb22 plasmid contains the entire rat leptin coding region. The leptin probe was obtained by amplifying the pOb22 plasmid with the following primers: 5'-CCAGCGAGGAAAATGTGCTG-3' and 5'-GGAATCGTG CGGATAACTTT-3'. The fragment was separated by agarose-gel electrophoresis and recovered by gel extraction (Qiaquick, Qiagen). The purified fragment was labeled with ^{32}P using the Rediprime labeling system (Amersham, Buckinghamshire, UK).

The 18S rRNA primer was synthesized using a Beckman 1000*M* oligonucleotide synthesis machine. The 18S sequence is as follows: 5-GACAAGCATATGCTACTGGC-3' *(33)*. The primer was end-labeled with ^{32}P using T4 polynucleotide kinase (Promega, Madison, WI). After labeling, the unincorporated nucleotide was purified through a Qiaquick nucleotide removal kit (Qiagen). Minor variations in loading of total RNA for experiments 1 and 3 were corrected by hybridization to the 18S primer after the blot was stripped off the leptin probe.

The human actin probe was obtained from Clontech. Twenty nanograms of the fragment was labeled with ^{32}P using the Rediprime labeling system (Amersham). Minor variations in loading of total RNA for experiment 2 were corrected by hybridization to actin cDNA after the blot was stripped off the leptin probe.

For hybridization to cDNA probes, the blots were prehybridized in 6X SSC (0.9*M* NaCl, 0.09*M* Na_3citrate·$2H_2O$), 50% formamide, 0.5% sodium dodecyl sulfate (SDS), and 100 µg/mL denatured, fragmented salmon sperm DNA for 8 h at 50°C. Denatured leptin or actin probes were then added to the prehybridization mix to a concentration of 0.5×10^6 cpm/mL and hybridized overnight at the same temperature. The blots were then washed twice in 2X SSC/0.1% SDS at room temperature for 15 min each and twice in 0.2X SSC/0.5% SDS at 50°C for 15 min each.

For hybridization to the 18S oligonucleotide probe, the blots were prehybridized in 6X SSC, 0.1% SDS, 100 µg/mL denatured salmon sperm DNA, and 5X Denhardt's solution for 8 h at 42°C before addition of probe to a concentration of 0.5×10^6 cpm/mL. The membranes were hybridized to the probe overnight at 42°C. After hybridization, the blots were washed twice in 6X SSC/0.1% SDS at room temperature for 15 min and once in 6X SSC/0.1% SDS for 30 min at 50°C.

The blots were exposed to Biomax MS autoradiographic film (Kodak, Rochester, NY). Relative leptin mRNA levels were quantitated by scanning densitometry (Bio-1D, v5.08, Vilber Lourmat) and small variations in sample loading were corrected against either 18S rRNA or actin mRNA levels.

The blots were stripped by shaking in boiling 0.5% SDS for 10 min followed by a 5 min-wash in 2X SSC after hybridization to the leptin cDNA probe. The blots were subsequently hybridized to either actin cDNA probe or the 18S oligonucleotide probe to correct for minor variations in loading.

Statistical Analysis

The means of the various treatment groups were compared using the one-way analysis of variance followed by Student Newman Keuls multiple comparisons test (SPSS for Windows v.6). Differences were considered significant at $p < 0.05$.

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