Partial Leptin Restoration Increases Hypothalamic-Pituitary-Adrenal Activity While Diminishing Weight Loss and Hyperphagia in Streptozotocin Diabetic Rats

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Chronic leptin administration at pharmacologic doses normalizes food intake and body weight in streptozotocin (STZ)-diabetic rats. We examined the metabolic effects of acute partial physiological leptin restoration in STZ-diabetic rats by using subcutaneous osmotic mini pumps. Groups: (1) Rats infused with vehicle (DV); (2) rats infused with recombinant murine methionine leptin (DL) at 4.5 $\mu g \cdot (kg\ body\ weight \cdot d)^{-1}$; (3)pair-fed rats (DP) given a food ration matching that consumed by the DL group. A fourth group of nondiabetic, normal (N) rats was also studied to assess normal metabolic efficiency, hypothalamic-pituitary-adrenal (HPA) activity and sympathoadrenal activity. Following leptin infusion, food consumption by DL rats was significantly lower than in DV rats. Paradoxically, despite a similar food intake to that of the DP group, which demonstrated a 40% reduction in body mass, DL rats increased their initial body weight by ~20% ($P < .05$). Plasma corticosterone and ACTH concentrations were elevated by 2-fold to 3-fold in DL versus N, DP, and DV rats. In the pars distalis, glucocorticoid receptor (GR) mRNA levels were significantly higher in DL and DP rats compared with N and DV rats. Our results suggest that partial restoration of physiologic leptin: (1) successfully reduces hyperphagia while allowing body weight gain in STZ-diabetic rats; (2) increases corticosterone levels in STZ-diabetic rats, which may in turn counteract the anorexic effects of diabetes; and (3) is associated with increased pituitary GR mRNA levels, despite elevated corticosterone levels, suggesting that leptin may interfere with the negative feedback regulation of the HPA axis.

LEPTIN LEVELS decrease dramatically in newly diagnosed patients with type 1 diabetes mellitus, as well as in patients suffering from lipodystrophic syndrome.[1-3] Streptozotocin (STZ)-diabetic animals, often used as a study model of type 1 diabetes, are characterized not only by decreased insulin levels and hyperglycemia, but also by a 5-fold to 10-fold decrease in circulating leptin levels.[4-6] It was previously suggested that this decrease in leptin underlies the hyperphagia often observed in diabetic animals.[6] Interestingly, chronic subcutaneous administration of leptin at pharmacologic doses results in partial correction of food intake, as well as complete normalization of postabsorptive plasma glucose levels.[7] A recent study by Miyanafa et al[8] has demonstrated that leptin cannot only improve the metabolic profile, but also diminishes insulin requirements for re-establishing normoglycemia in STZ-diabetic mice.

The hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system are known to be regulated by leptin.[9,10] While some studies suggest an inhibitory role of leptin on HPA activity,[11] others suggest that leptin may act as a positive regulator of the HPA axis.[12,13] Interestingly, leptin appears to be more effective in stimulating ACTH and glucocorticoid secretion when administered at low concentrations in vivo.[14] Moreover, leptin was shown to act directly on dispersed adrenal cells in vitro as evident by the increase in glucocorticoids release.[15] It is possible, therefore, that physiologic concentrations of leptin increase HPA activity.

It appears that glucocorticoids and leptin counteract each other's effects on food intake and body weight maintenance. Corticosterone removal by adrenalectomy leads to a marked amplification of the anorexic effects of leptin, resulting in severe hypophagia and weight loss.[16] Dexamethasone treatment or corticosterone replacement (at levels observed during stress) can substantially attenuate the effects of leptin in rodents; restoring normal food consumption and preventing weight loss.[16,17] As for sympathetic activity, an increase in epinephrine and norepinephrine levels is often observed following leptin administration.[9] This increase in sympathetic activity is thought to play a pivotal role in the ability of leptin to increase basal metabolic rate and promote thermogenesis.[9] These widespread effects underline the important role of leptin as a pivotal regulator of the metabolic profile in insulin-deficient diabetic rats.

The purpose of this study was to examine whether partial restoration of physiologic levels of leptin, by constant subcutaneous infusion, can improve the metabolic state. Such an improvement is associated with the alteration of basal HPA and sympathetic activity in STZ-diabetic rats.

MATERIALS AND METHODS

Experimental Animals

Male Sprague Dawley rats (Charles River Laboratories, Quebec, Canada) weighing between 300 and 350 g were individually housed in opaque cages and were acclimatized to the animal facility (7 to 14 days) prior to the start of experimentation. Animals were fed rat chow (Ralston Purina, St Louis, MO) and were allowed free access to water.

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The experiments described below were performed according to protocols approved by the Animals Care committee at the University of Toronto, in accordance with guidelines set by the Canadian Council for Animal Care.

On day 0, indwelling Silastic tubing (length 3 cm; inner diameter, 0.020 in; outer diameter, 0.037 in.; Dow Corning, Midland, MI) connected to a polyethylene catheter (length 10 cm; PE-50, Clay Adams, Boston, MA) was inserted into the left carotid artery under anesthesia, as described previously.[18] Diabetes was induced (day 0) with a single intraperitoneal injection of STZ (65 mg/kg; Sigma, St Louis, MO); normal nondiabetic rats received a saline injection. Animals treated with STZ were given 10% sucrose in their drinking water for the first 24 hours following STZ injection to prevent hypoglycemia.[19] This model of type 1 diabetes mellitus features moderate diabetes with fasting and fed hyperglycemia and normal plasma insulin during fasting, but markedly reduced plasma insulin during feeding.[18,20] Blood glucose was monitored twice daily in both normal and diabetic rats, using a blood glucose meter (Glucometer Elite 3909; Bayer, Etobicoke, Ontario, Canada), to ensure that normoglycemia was maintained in controls and that adequate hyperglycemia (>15 mmol/L) was achieved in the fed state in diabetic rats.

On day 4, animals were divided into 3 groups. Group 1: normal nondiabetic rats (N, n = 13) infused with vehicle-loaded (phosphate-buffered saline) osmotic mini pumps (Alzet; Durect, Cupentino, CA) for 4 days. Group 2: diabetic vehicle treated rats (DV, n = 10) infused with vehicle-loaded osmotic mini pumps for 4 days. Group 3: diabetic rats infused for 4 days with recombinant murine leptin (DL, n = 9) (Sigma) at 4.5 $\mu g \cdot (kg\ body\ weight \cdot d)^{-1}$ using subcutaneous osmotic minipumps. Group 4: diabetic pair-fed rats (DP, n = 10), receiving a similar food ration to that consumed by the DL group, were infused with vehicle-loaded osmotic mini pumps for 4 days. The purpose of the DP group was to control for the influence of leptin on decreased food consumption, independent of other physiologic effects of leptin replacement.

On day 7, blood was sampled via carotid catheter exteriorized outside of the rodent cage while the rats were in the fed state (10 AM to 11 AM). Rats were then fasted for 24 hours to examine the effect of leptin on fasting blood glucose and hormone levels. On the morning of day 8, rats were rapidly removed from their cages and euthanized by decapitation between 10 AM and 11 AM. Trunk blood samples were taken from each animal and centrifuged immediately. Plasma was transferred into tubes for storage at −20°C (or at −80°C for catecholamines) and subsequent analysis. Pituitary glands were quickly removed under sterile conditions, frozen on dry ice, and stored at −80°C until processing for in situ hybridization.

Determination of Plasma Hormone and Catecholamine

Plasma insulin was measured using a previously characterized[19] modified version of the insulin radioimmunoassay (RIA) by Herbert et al.[21] Plasma leptin (Linco Research, St Charles, MO), ACTH (ICN Pharmaceuticals, Orangeburg, NY), and corticosterone (ICN Pharmaceuticals, Orangeburg, NY) concentrations were determined using commercially available RIA kits.[19] Epinephrine and norepinephrine levels were determined using the simultaneous single-isotope derivative radioenzymatic assay (Amersham Biosciences, Piscataway, NJ).[19]

In Situ Hybridization

The method of in situ hybridization has been described in detail, previously.[22] Coronal pituitary cryosections (10 μm) were obtained. The sections were then thaw-mounted onto (poly)-L-lysine (Sigma)-coated slides, fixed in phosphate-buffered paraformaldehyde (4%) and dehydrated prior to hybridization.

As described previously, proopiomelanocortin (POMC) (bases 572 to 616),[23] and GR (bases 1,321 to 1,365)[24] oligonucleotide probes,[19] were labeled using terminal deoxynucleotidyltransferase (Pharmacia Biotech, Baie d'Urfé, Québec, Canada) and [^{35}S]-deoxyadenosine 5′-(α-thio)triphosphate (1,300 Ci/mmol, NEN Life Science Products, Du Pont Canada, Mississauga, Ontario, Canada) to a specific activity of 1.0×10^9 cpm/μg. Labeled probe in situ hybridization buffer (180 μL) was applied to each slide at a concentration of 1.0×10^6 cpm/μL. Slides were incubated overnight in a moist chamber at 42.5°C. After washing in 1× SSC (20 minutes at room temperature), 1× SSC (35 minutes at 55°C), the slides were rinsed twice with 1× SSC and with 0.1× SSC at room temperature, then dehydrated in 70% and 95% ethanol, air dried, and exposed to autoradiographic film (Biomax; Eastman Kodak, Rochester, NY). The films were developed using standard procedures (exposure time: POMC, 2 hours and GR, 6 days).

Pituitary sections were processed simultaneously for each probe to allow for direct comparisons between the 4 groups. Six sections were selected from each animal to be analyzed by in situ hybridization. The sections were exposed, together with ^{14}C-standards (American Radiochemical, St Louis, MO) to ensure analysis in the linear region of the autoradiographic film. The relative optical density (ROD) of the signal on autoradiographic film was quantified, after subtraction of background values, using a computerized image analysis system (Imaging Research, St Catherines, Ontario, Canada).

Statistical Analysis

Hormone data are presented as mean ± SEM, and in situ hybridization data are expressed as ROD (mean ± SEM). Statistical analysis was performed with Statistica 6.0 software (StatSoft, Tulsa, OK). Comparisons of data among the 4 different groups were made using analysis of variance (ANOVA) in conjunction with Fisher's LSD test. In all tests, significant differences were presumed at $P < .05$.

RESULTS

Plasma Leptin and Plasma Insulin Concentrations

Compared with controls, STZ treatment resulted in a 4-fold lower ($P < .001$) plasma insulin concentration in the fed state in all 3 diabetic groups. Fasting plasma insulin levels were similar, however, among all groups (Fig 1A). Leptin concentrations demonstrated a similar trend to that of insulin and, in the fed condition, were significantly ($P < .001$) lower in the DV and DP rats when compared wtih the N and DL groups (Fig 1B). Following a 24-hour fast, plasma concentrations of leptin were higher in the DL group compared with the N, DP, and DV groups.

Blood Glucose, Food Intake and Body Weight Change

All 3 diabetic groups demonstrated marked ($P < .0001$) hyperglycemia following STZ injection under the fed condition when compared with normal controls (Fig 2). Fasting blood glucose levels were also elevated ($P < .0001$) in the DV group, but were normalized in the DP and DL groups. Food intake was significantly ($P < .02$) higher in the DV group compared with the DL and N groups (Table 1). Despite similar food intake, DP rats had a 40% decrease ($P < .005$) in body weight when compared with the DL rats (Table 1). To better illustrate these effects, we calculated the ratio between food intake and body weight relative to nondiabetic controls. In agreement with food intake and body weight changes, DV rats demonstrated a significant increase ($P < .02$) in food intake, while the DL group showed no statistical differences when compared with N rats (Fig 3).

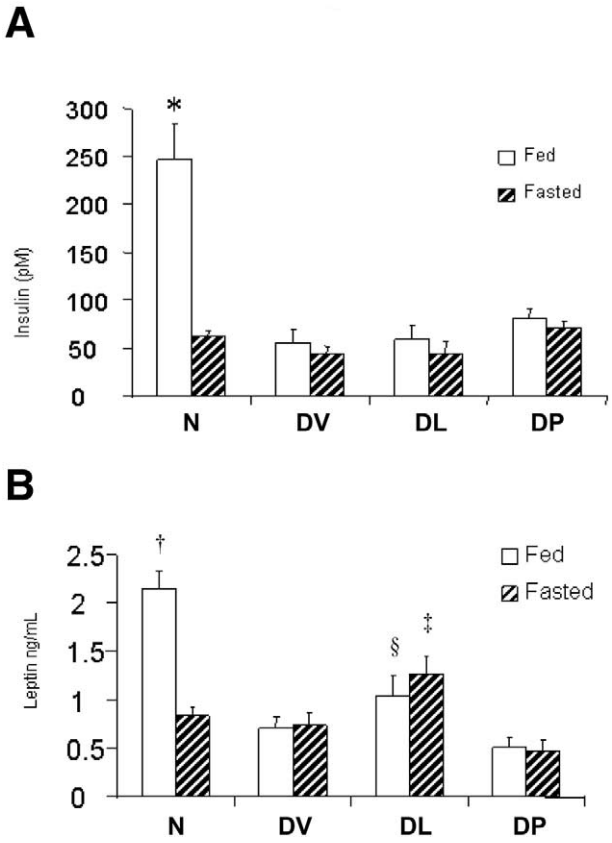

Fig 1. (A) Plasma insulin and (B) leptin concentrations in normal (N), diabetic (DV), diabetic leptin-treated (DL), and diabetic pair-fed (DP) rats under fed (open bars) and fasted (hatched bars) states. Values are expressed as mean ± SEM. *$P < .02$ *v* DV, DL, and DP; †$P < .001$ *v* DV, DL, and DP; §$P < .05$ *v* DV and DP; ‡$P < .03$ *v* N, DV, and DP.

Table 1. Food Consumption and Body Weight Change of N, DV, DL, and DP Rats

Group	Food Intake (g/kg BW)	BW Change (% of initial BW)
N (n = 13)	67.4 ± 2.9	138 ± 5
DV (n = 10)	97.0 ± 5.6†‡	122 ± 14
DL (n = 9)	77.7 ± 7.7*	119 ± 11
DP (n = 10)	80.7 ± 1.4	59 ± 13§

NOTE. Data are expressed as mean ± SEM.

Abbreviations: N, normal; DV, diabetic; DL, diabetic leptin; DP, diabetic pair = fed; BW, body weight.

*$P < 0.008$ *v* DV.

†$P < .02$ *v* DP.

‡$P < .0001$ *v* N.

§$P < .005$ *v* DL.

Plasma ACTH and Corticosterone Concentrations

In the fed state, plasma ACTH levels were similar among all groups (Fig 4A). In the fasted state, however, plasma ACTH levels were significantly ($P < .05$) higher in the DL group than in the other 3 groups (Fig 4A). As expected, normal rats demonstrated low corticosterone levels under both fed and fasted conditions (Fig 4B). In the fed state, DL rats exhibited 3-fold to 4-fold higher corticosterone levels than the other groups ($P < .001$, Fig 4B). Fasting resulted in increased ($P < .03$) corticosterone levels in the DP rats, while all other groups exhibited no significant differences between fed and fasted corticosterone levels.

Plasma Epinephrine and Norepinephrine Concentrations

Basal plasma norepinephrine levels were markedly ($P < .006$) increased in DV rats when compared with both N and DL animals and showed a tendency ($P < .08$) towards an increase in DP rats compared with normal rats (Fig 5A). In contrast, epinephrine levels were significantly ($P < .01$) increased in leptin-treated diabetic rats when compared with pair-fed diabetic rats (Fig 5B).

Pituitary POMC and Glucocorticoid Receptor mRNA Levels

POMC mRNA levels in the pars distalis and pars intermedia were not different among the 4 groups (Fig 6A). However, glucocorticoid receptor (GR) mRNA levels in the pars distalis were significantly ($P < .05$) increased in both DP and DL animals when compared with both N and DV animals (Fig 6B).

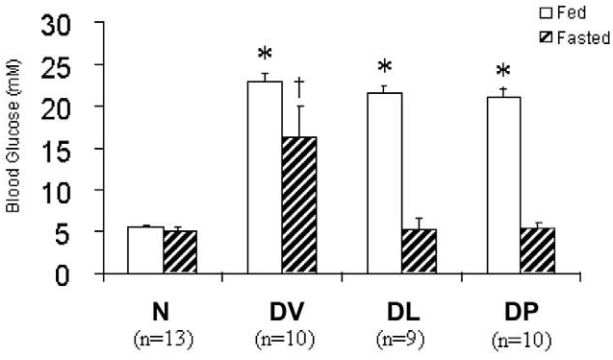

Fig 2. Blood glucose levels in N, DV, DL, and DP rats under fed (open bars) and fasted (hatched bars) states. Values are expressed as mean ± SEM. *$P < .0001$ *v* N. †$P < .0001$ *v* N, L, and DP.

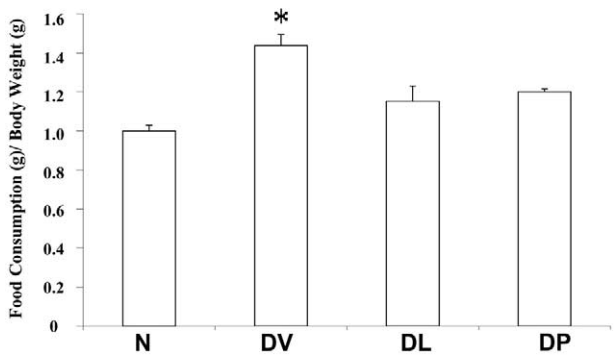

Fig 3. Food intake to body weight ratio in DV, DL, and DP rats relative to N rats . Values are expressed as mean percentiles ± SEM. *$P < .03$ *v* DL, N, and DP.

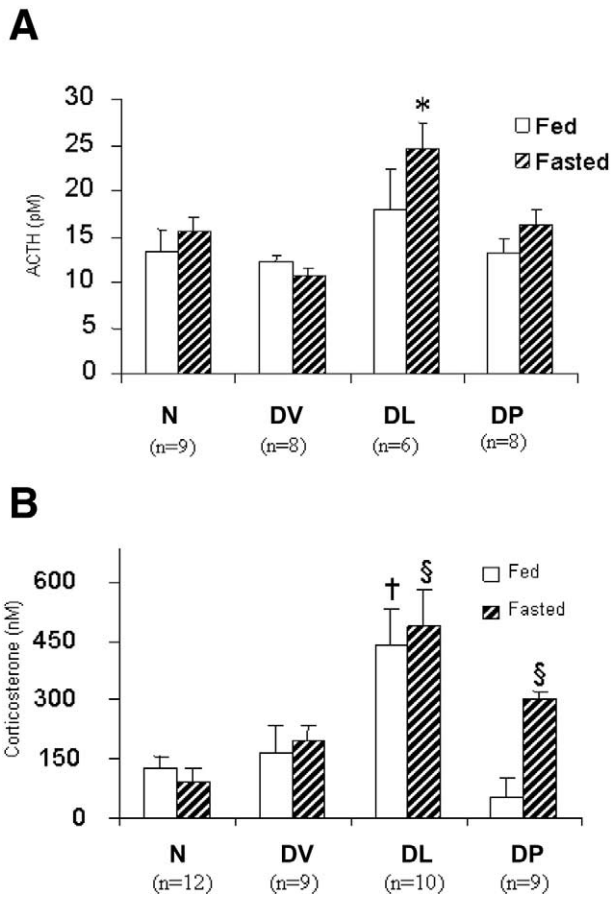

Fig 4. (A) Basal plasma ACTH and (B) corticosterone concentrations in N, DV, DL, and DP rats under fed (open bars) and fasted (hatched bars) states. Values are expressed as mean ± SEM. *$P < .05$ *v* N, D, and DP; †$P < .001$ *v* N, D, and DP; §$P < .03$ *v* N; ‡$P < .03$ *v* DP.

DISCUSSION

This study was designed to examine the effects of partial restoration of circulating leptin on metabolic efficiency and basal HPA activity in STZ-diabetic rats. By infusing leptin at a very low rate, we demonstrated that even minimal constant amounts of leptin have a dramatic effect on food intake and body mass in STZ-diabetic rats. More specifically, to our surprise, a small amount of leptin not only lowered food intake in STZ-diabetic rats, but paradoxically prevented the weight loss associated with pair feeding per se. In addition, leptin infusion activated basal HPA activity in STZ-diabetic rats, despite increased expression of GRs in the anterior pituitary. Moreover, we found that the dosage of leptin used in our study was associated with an increase in epinephrine and a decrease in norepinephrine concentrations. These observations emphasize the paradoxical role of leptin in type 1 diabetes, demonstrating a beneficial effect of leptin on overall metabolic state, but also a detrimental effect by increasing HPA activity.

Rats treated with a single medium dose of STZ are hyperglycemic, insulin resistant, and show a significant decrease in plasma leptin concentrations. As opposed to high-dose STZ-treated rats, these animals do not require insulin support for short-term survival, because basal insulin secretion is usually maintained.[25] It was previously suggested that leptin dysregulation may partially account for the overall deterioration of the metabolic state in these animals.[7] Chinookoswong et al[7] demonstrated that leptin administration in pharmacologic doses can normalize blood glucose levels, while allowing the animal to maintain its initial body mass. Similarly, in our study, DV rats had a 40% increase in food intake when compared with nondiabetic controls (Table 1). Leptin infusion reduced food consumption in diabetic rats, thereby suggesting that the hyperphagia associated with type 1 diabetes is caused by low circulating leptin concentrations. Surprisingly, although eating less, leptin-treated animals were able to maintain a similar body weight to that of diabetic vehicle-treated rats and controls. To better illustrate these observations, we compared food intake to body weight ratio in diabetic rats with normal controls. This ratio was then used as an indicator for diabetes-induced hyperphagia. As expected, food intake to body weight ratio was markedly higher in vehicle diabetic animals compared with normal controls and was partially corrected following leptin infusion. This

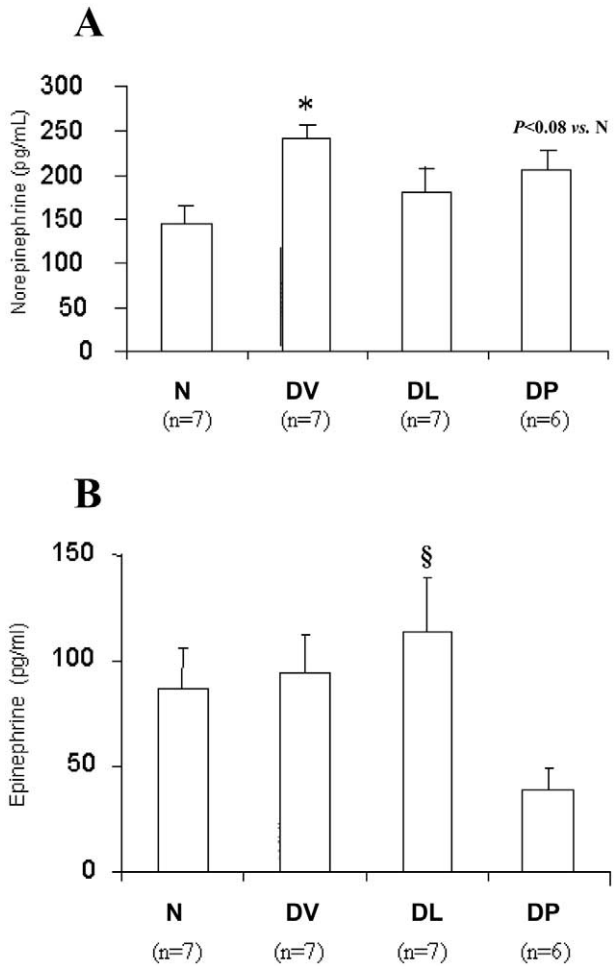

Fig 5. (A) Fed basal plasma norepinephrine and (B) epinephrine concentrations in N, DV, DL, and DP rats. Values are expressed as mean ± SEM. *$P < .006$ *v* N, DL. §$P < .01$ *v* DP.

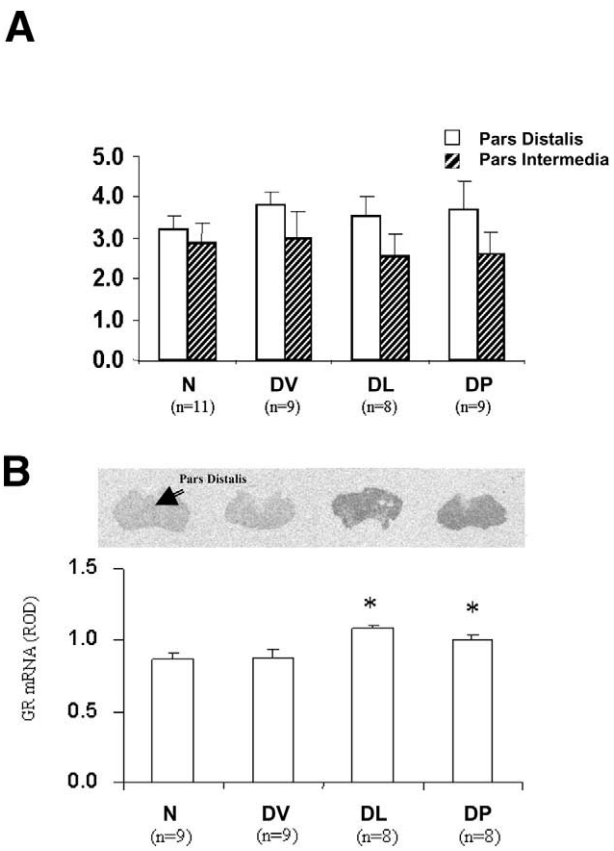

Fig 6. (A) Anterior pituitary POMC and (B) GR mRNA levels in N, DV, DL, and DP rats. Values are expressed as mean ± SEM. *$P < .006$ *v* N and DV.

current study suggests that a modest steady elevation in circulating leptin can produce beneficial effects in STZ-diabetes as evident by the rats' ability to better preserve their body mass despite a decrease in food consumption and low basal insulin levels. Further investigation using direct and indirect calorimetry and tissue analysis will be required to provide a better understanding of the mechanisms underlying these observations.

In the present study, corticosterone levels were increased in ad libitum-fed diabetic rats, however; this increase did not reach statistical significance (Fig 4B). Nonetheless, the observation that fasting corticosterone levels were more than 2 times higher in all diabetic groups compared with normal animals recapitulates our previous study, which demonstrated increased HPA activity in STZ-diabetic rats.[26] Fasting resulted in a significant increase in basal corticosterone concentrations in diabetic pair-fed rats. In contrast, ACTH remained unchanged under fed and fasting conditions in both diabetic ad libitum and diabetic pair-fed rats. Several groups have shown that diabetes, food restriction, and food deprivation can increase HPA activity.[26-30] Insulin treatment not only restores euglycemia, but also normalizes the concentrations of both corticosterone and ACTH.[26] In our study, fasted corticosterone levels were significantly elevated in pair-fed diabetic rats. This increase may reflect the more profound negative energy balance and a poor metabolic state in pair-fed rats, which is produced by the combination of food restriction and untreated diabetes. This increase was observed despite normal fasting glucose levels, indicating that overnight glucose normalization is insufficient in reducing HPA activity. Taken together, these observations suggest that food restriction per se imposes a moderate stress that does not increase corticosterone concentrations under nonfasted conditions; however, when followed by an overnight fast, can result in a marked increase in basal corticosterone levels in STZ-diabetic rats. Our inability to demonstrate a significant increase in ACTH and corticosterone levels in diabetic vehicle-treated groups may be attributed to the pulsatile secretion of ACTH and to the fact that in the current study we measured ACTH at a single time point. These 2 factors contributed to high variability within the groups.[28,31]

Many lines of evidence suggest a mutual relationship between leptin and the HPA axis.[10] Leptin administration in physiologic doses can act as a potent stimulator of HPA activity.[12-14] In the present study, HPA activity increased following leptin infusion in diabetic rats. Because rats are nocturnally active animals, both ACTH and corticosterone levels gradually decline during the dark phase, reaching a nadir at the onset of the light phase, and remaining low during the light phase until later in the afternoon.[32,33] In our study, leptin treatment markedly increased both corticosterone and ACTH concentrations in diabetic rats even during the light phase (Fig 4). To address the increased HPA activity in leptin-treated animals at the molecular level, we examined both POMC and GR mRNA levels in the anterior pituitary. GRs in the anterior pituitary play an important role in glucocorticoid negative feedback regulation of the HPA axis.[34] In our previous study, GR levels in the pituitary remained unchanged in diabetic animals, but were increased following insulin treatment.[19] Similarly, in the present study, no increase was detected in GR mRNA in diabetic vehicle-treated rats, while leptin infusion resulted in a small, but significant, increase in pituitary GR mRNA levels. Therefore, it may be that the increase in pituitary GR mRNA associated with insulin treatment in diabetes is mediated by insulin's increase in leptin concentration. Increased GR mRNA levels in the pars distalis, which would suggest an increase in glucocorticoid negative feedback, are not associated with any change in pituitary POMC mRNA expression or with decreased plasma ACTH or corticosterone levels. It could be that leptin interferes with mechanisms involved in negative feedback, because increased expression of GR could not normalize the elevations in ACTH and corticosterone levels. Nevertheless, we cannot exclude the possibility that GRs that are present on noncorticotropes, notably growth hormone cell, may be also affected by leptin in a way that would alter the expression of a GR in these cells.

When in excess, glucocorticoids are associated with increased appetite, most likely through their permissive effect on neuropeptide Y.[35,36] Furthermore, the loss of body weight and decreased food consumption, which is observed in adrenalectomized mice, can be completely reversed by glucocorticoid replacement.[37] In contrast, leptin administration results in a profound decrease in both food consumption and body weight in normal animals.[38] The anorexic effects of leptin are further augmented in adrenalectomized rats when compared with nor-

mal rats.[16] Dexamethasone administration to leptin-treated adrenalectomized rats significantly dampens the anorexic effects of leptin, thus preventing body weight loss and normalizing food intake.[16] Furthermore, high corticosterone levels can completely abolish the effects of leptin in adrenalectomized mice.[17] These findings suggest that glucocorticoids can counteract the physiologic effects of leptin on both food consumption and body weight change. In our study, leptin-treated diabetic rats showed increased basal corticosterone levels under both fed and fasted conditions. We postulate that an acute increase in corticosterone may offset some of the negative effects on food consumption that are associated with leptin infusion, thus preventing underfeeding and excessive weight loss.

The observed differences in sympathetic activity as indicated by decreased and increased fed norepinephrine and epinephrine levels, respectively, under fed conditions in leptin-treated rats are difficult to interpret, because the higher norepinephrine levels in DV rats may be a result of dehydration. Nevertheless, the normalization of norepinephrine following leptin may contribute to the overall improvement in the metabolic state observed in this group, because increased sympathetic activity can induce higher metabolic activity. Further studies using continuous blood sampling will be required to better assess sympathetic activity for more extended periods of time.

In summary: leptin can act as a stimulator of HPA activity, resulting in elevated plasma ACTH and corticosterone levels. This increase in pituitary-adrenal activity is observed despite elevated mRNA levels of GRs, indicating that leptin may interfere with negative feedback regulation. Because corticosterone can act as an orexigenic agent, its increase following leptin treatment may counteract the direct anorexic effects of leptin, further contributing to improved metabolic efficiency following low-dose leptin treatment in STZ-diabetic rats. The significance of leptin's effect on sympathetic activity needs further exploration.

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