EXPERIMENTAL STUDY

Effect of leptin on ACTH-stimulated secretion of cortisol in rhesus macaques and on human adrenal carcinoma cells

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Abstract

Objective: Because glucocorticoids stimulate leptin release and, at least *in vitro*, leptin inhibits cortisol secretion, a feedback system between glucocorticoids and leptin has been proposed. However, in humans and non-human primates there are no *in vivo* studies to support any role for leptin in the control of the hypothalamic–pituitary–adrenal axis. In this study, we investigated the effect of leptin on (i) ACTH-stimulated secretion of cortisol in six male rhesus monkeys and (ii) basal and forskolin (FSK)-stimulated cortisol secretion by the human adrenal carcinoma cell H295R *in vitro*.

Design and Methods: *In vivo* studies: after suppression of endogenous ACTH with either dexamethasone ($n = 6$) or a corticotropin-releasing factor (CRF) antagonist (D-Phe CRF(12–41)) ($n = 3$), 1 μ g bolus of human ACTH(1–24) was administered to stimulate adrenal cortisol release. Blood samples were collected every 15 min for 3 h. Leptin (1 mg) was infused over 4 h, starting 1 h before ACTH bolus. *In vitro* studies: NCI-H295R cells were incubated for 6, 12, 24 and 48 h in the absence or presence of 20 μ M FSK in combination with leptin (100 ng/ml medium). Cortisol levels in serum and medium were measured by solid phase radioimmunoassay.

Results: Acute leptin infusion to rhesus monkeys did not change basal cortisol levels, peak cortisol levels after ACTH(1–24) or the area under the curve when compared with studies in which leptin was not given. FSK increased cortisol levels in medium at 24 and 48 h, but leptin did not change cortisol release in either control or FSK-stimulated cells.

Conclusions: Short-term leptin infusion affected neither the cortisol response to ACTH in non-human primates *in vivo* nor cortisol release (basal or FSK stimulated) by H295R cells, *in vitro*. These data suggest that leptin may not be an acute regulator of primate adrenal cortisol secretion.

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Introduction

Glucocorticoids participate in the control of caloric intake and adipogenesis. In patients who have elevated glucocorticoids such as in Cushing's disease, obesity or weight gain is observed in 79–95% of the affected individuals (1). Glucocorticoids also stimulate food intake (2), synthesis and activity of the adipose tissue lipoprotein lipase (3) and adipocyte differentiation and distribution (4, 5). Some of these actions are in synergism with insulin (6, 7). Glucocorticoids also participate in epinephrine synthesis in the adrenal medulla (8) and both epinephrine and insulin influence energy metabolism.

In mouse and rat experimental models, obesity does not progress after adrenalectomy (9). In the *ob/ob* mouse, obesity is caused by a mutation in the gene for leptin (10), a protein that influences energy intake and expenditure (11) at both central (12) and peripheral

sites (13, 14). Glucocorticoids stimulate the synthesis and release of leptin in rodents and humans (15–18) and reciprocally, the hyperglucocorticoidemia observed in *ob/ob* mice is reduced with leptin administration (19). Leptin blocks HPA axis activation in restrained mice and, depending on the circumstances, can block corticotropin-releasing hormone (CRH) release *in vitro* (20). Leptin also decreases basal cortisol release by bovine adrenal cells (21) and adrenocorticotropin (ACTH)-stimulated cortisol release by bovine (21) and human adrenal cells (22) *in vitro*. Together, these data suggest an inhibitory feedback action of leptin on the hypothalamic–pituitary–adrenal axis.

The two objectives of this study were to determine if leptin affects ACTH-stimulated secretion of cortisol in rhesus monkeys *in vivo* and to determine if leptin alters cortisol secretion by human adrenal carcinoma cells *in vitro*.

Materials and methods

In vivo studies

Six young male rhesus macaques (*Macaca mulatta*), 5.5–8 kg in weight (3–4.5 years old), were housed in individual cages under temperature ($23 \pm 2^\circ\text{C}$)- and light-controlled conditions (lights on 0600–1800 h). Animals were maintained on a diet of monkey chow and fresh fruit with tap water available *ad libitum*. Routine daily animal care and maintenance consisted of feeding from 0700–0900 h, cage cleaning from 0900–1000 h, and an unrestricted afternoon feeding at 1300 h. Fresh fruit was provided three times a week. Entry into the primate room was restricted to the animal caretakers and personnel involved in the research project. All animal studies were approved by the Institutional Animal Care and Use Committee at Texas Tech University Medical Center.

Each animal experienced two studies separated by at least 48 h. In the control study, cortisol secretion was evaluated after i.v. administration of a 1 μg bolus of human ACTH(1–24) (Bachem Inc. Fine Chemicals, Torrance, CA, USA). In the experimental study, 1 mg recombinant human leptin (R&D Systems, Inc., Minneapolis, MN, USA) was infused for 4 h beginning 1 h before ACTH administration. We have found in other studies that this level of leptin infusion (250 g/h) is sufficient to elevate circulating levels of leptin to the high range observed for adult macaques. The leptin bioactivity was documented by its ability to lower body weight in 6-week-old mutant obese C57BL/6J mice (*ob/ob*) (Jackson Laboratory, Bar Harbor, ME, USA) when administered intraperitoneally for 2 days (2 $\mu\text{g/g/12 h}$).

In six animals studies were initiated in the morning, with blood samples collected every 15 min between 0745 and 1100 h. Leptin was infused between 0700 and 1100 h and ACTH(1–24) was administered immediately after the 0800 h sample. To achieve a homogeneous basal cortisol level at the beginning of the study, a 1 mg i.v. bolus of dexamethasone (DXM; Sigma–Aldrich, St Louis, MO, USA) was given at midnight the night before the study.

Three additional animals were studied in the evening with blood samples collected every 15 min between 1845 and 2200 h. Leptin was infused between 1800 and 2200 h and ACTH(1–24) was administered immediately after the 1900 h sample. The corticotropin-releasing factor (CRF) antagonist, D-Phe CRF(12–41) (Bachem Inc. Fine Chemicals) was infused (100 $\mu\text{g/h}$) from 1700 to 2200 h to suppress endogenous ACTH secretion.

Collection of blood samples from unrestrained animals was accomplished as previously described (23). The samples were centrifuged at the end of the study and the plasma was withdrawn and stored frozen at 20°C in polypropylene vials until assays were performed.

In vitro studies

Human NCI-H295R adrenal tumor cells (WE Rainey, UT Health Sciences Center, Dallas, TX, USA) (24) were grown in Dulbecco's modified Eagle's and Ham's F12 media 1:1 (v/v) (DMEM–F12) supplemented with pyridoxine HCl, L-glutamine, and 15 mmol/l HEPES. Complete DMEM–F12 was prepared by adding 1% (v/v) ITS Plus (Collaborative Biomedical Products, Bedford, MA, USA – containing 6.25 μg insulin/ml, 6.25 μg transferrin/ml, 6.25 μg selenium/ml, 1.25 mg bovine serum albumin, and 5.35 μg linoleic acid/ml), 1% (v/v) penicillin/streptomycin (Gibco-BRL, Gaithersburg, MD, USA), 0.01% (v/v) gentamicin (Sigma Chemical Company, St Louis, MO, USA) and 2% (v/v) Ultrosor G (BioSeptra SA, Villeneuve la Garenne Cedex, France).

In the present studies, H295R adrenocortical cells grown in 150 cm^2 flasks (Falcon, Lincoln Park, NJ, USA) were incubated at 37°C in a humidified atmosphere containing air/carbon dioxide (95%/5%, v/v). The medium was changed every 3 days and cells were subcultured with 0.25% (w/v) trypsin every 7 days (split ratio, 1:2). To remove trypsin, the cell suspension was mixed with horse serum (1:2, v/v), centrifuged for 10 min at 700 g, then resuspended in complete DMEM–F12 and plated.

Cells used for experiments were subcultured from 60% confluent stock cultures into Nunc six-well culture plates 24 h before they were used. Each well was washed with 2 ml phosphate-buffered saline (PBS) before being pre-incubated for 1 h in 2 ml complete DMEM–F12. After discarding the pre-incubation medium, each well was again washed with 2 ml PBS. Sequential 6, 12, 24 and 48 h experimental incubations were conducted on each set of plates with 2 ml complete DMEM–F12 in the absence or presence of 20 $\mu\text{mol/l}$ forskolin (FSK, Sigma Chemical Company) in combination with recombinant human leptin (100 ng/ml medium). To verify that these cells were responsive to ACTH and that the steroidogenic enzymes were functional, several experiments were also conducted with ACTH(1–24) or 20α -dihydroxycholesterol instead of FSK (results not shown). At the end of each incubation period, the medium was removed from each well and placed in glass storage vials, each well was washed with 2 ml PBS, and combined with the appropriate medium sample. Two milliliters of medium containing the appropriate experimental treatment was then replaced in each well and the incubation was continued. Samples in vials were frozen until assayed for cortisol. Each experimental treatment was replicated six times and the experiments were repeated four times. Leptin concentration was measured in media after 48 h incubation.

Assays Serum cortisol was measured in duplicate by solid phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). Leptin was

measured in duplicate by radioimmunoassay (Linco Research, Inc, St Louis, MO, USA).

Data analyses The paired *t*-test was used to compare ACTH-stimulated cortisol secretion in rhesus monkeys with and without leptin infusion. The variables compared were basal cortisol levels (pre-ACTH(1–24) infusion), peak response after ACTH(1–24) and area under the curve. Data from the *in vitro* study were analyzed by repeated measures ANOVA with *post hoc* comparison by Fisher's method.

Results

In vivo studies

With DXM suppression of the hypothalamic–pituitary–adrenal (HPA) axis, there was no difference between groups in basal (pre-ACTH(1–24)) serum cortisol levels at 0800 h (mean $\pm$ S.E.M.; 2.04 ± 0.71 μ g/dl control vs 1.97 ± 0.48 μ g/dl leptin). The cortisol peak response after ACTH(1–24) was achieved at 30 min in both groups (Fig. 1; 17.00 ± 2.60 μ g/dl control vs 15.57 ± 2.08 μ g/dl leptin). There were also no differences in the mean cortisol level between control and leptin-treated groups at any time point after leptin administration or in the area under the curve (1809.7 ± 496.47 μ g/dl min control vs 1750.93 ± 442.30 μ g/dl min leptin).

In the group treated with the CRF antagonist, D-Phe CRF(12–41), there were too few animals for statistical analysis; however, the cortisol profile was similar in the absence and presence of leptin (Fig. 2). The statistical analysis of combined data (group treated with DXM and group treated with D-Phe CRF(12–41) compared with controls) indicated there were no significant effects of leptin treatment (1712.6 ± 555 μ g/dl min control vs 1731.5 ± 562.5 μ g/dl min leptin).

Figure 1 Cortisol levels in adult male rhesus macaques after a 1 μ g i.v. bolus of ACTH(1–24). In both the control and experimental (Leptin; 1 mg infused over 4 h) trials, the animals were treated with dexamethasone (1 mg) at midnight before the study to suppress endogenous ACTH release.

Figure 2 Cortisol levels in two adult male rhesus macaques after a 1 μ g i.v. bolus of ACTH(1–24). In both the control and experimental (Leptin; 1 mg infused over 4 h) trials, the animals were treated with a CRF receptor antagonist (D-Phe CRF(12–41); 100 μ g/h infused over 5 h) to suppress endogenous ACTH release.

In vitro studies

Cells incubated with FSK secreted significantly more cortisol than control cells at 24 h and 48 h (Fig. 3). However, the addition of leptin into the incubation medium did not change cortisol release in either control or FSK-treated cells.

Discussion

As has been reported in the human (25), 1 mg DXM i.v. blocked ACTH-stimulated cortisol secretion after 8 h in the present study in male rhesus monkeys. Likewise, 1 μ g ACTH(1–24) stimulated a robust adrenal cortisol release in male macaques. However, the maximum response was less than that observed in human subjects (26). This could be due to lower sensitivity of monkey adrenal to human ACTH or, more probably, to a loss of ACTH during the infusion as has been described recently (27).

Short-term leptin infusion had no effect on *in vivo* cortisol release after ACTH infusion in DXM-suppressed monkeys. Since leptin is more effective in causing

Figure 3 Cortisol release by NCI-H295 human adrenal carcinoma cells. Forskolin (FSK) increases cortisol secretion vs control (* $P < 0.05$) at 24 h and 48 h. Leptin did not change cortisol release in either control or FSK-treated cells.

weight loss and reducing food intake in adrenalectomized rats than in intact rats and in adrenalectomized rats given DXM (28), it is possible that DXM caused leptin resistance and reduced the effect of leptin. However, in the present study, suppression of the HPA axis with a CRF antagonist, D-Phe CRF(12–41), rather than with DXM did not change the response to ACTH, indicating that the absence of a leptin effect was not due to glucocorticoid-mediated leptin resistance.

Also, leptin did not affect basal or FSK-stimulated cortisol release by human H295R adrenal tumor cells during a 48-h incubation period. The absence of a leptin effect on basal cortisol release agrees with previous studies with primary human adrenal cells (22, 29). However, leptin suppressed basal cortisol secretion in bovine adrenal cells (21) and contradictory leptin effects were observed during studies examining basal corticosterone secretion by rat adrenal cells. Increased basal secretion occurred with leptin treatment in one study (30) while no effect was found in another (22).

The reasons for the disparity between our results and the effects measured during previous studies are unknown. The leptin used in these experiments exhibited bioactivity and was shown to be present in the medium by RIA analysis at the end of the experiments. H295R adrenal cells were incubated with FSK instead of ACTH because we previously observed that ACTH is a weak stimulator of cortisol release in those cells (31). FSK was chosen as the stimulant to elevate H295R intracellular cAMP and cortisol secretion to avoid the suggestion that leptin effects were obscured by reduced ACTH responsiveness. If leptin does affect cortisol release from H295R cells *in vitro*, this effect does not involve FSK-stimulated intracellular cAMP production. It is not known whether the inhibitory action of leptin on cortisol release requires ACTH stimulation rather than FSK stimulation. Another explanation for the observed differences between normal and tumor cells

could include differences in leptin receptors. However, it has recently been shown that H295R cells express the full length Ob receptor mRNA and protein and that leptin does not regulate tumor cell proliferation (32). Leptin receptors are also expressed in the cortex of the human adrenal (29).

In accordance with our data, there are several findings suggesting that leptin does not exert a direct effect on the adrenal cortex *in vivo*. In the only two reports concerning congenital human leptin deficiency (33, 34), the affected individuals did not have elevated plasma cortisol levels. Licinio *et al.* (35) found that the 24-h patterns of circulating human leptin are inverse of those for ACTH and cortisol suggesting that leptin might suppress the adrenal function. However, the diurnal rhythm of leptin is entrained to meal pattern and phase shifts in plasma leptin are apparent within hours of changing the meal pattern. These phase shifts are not correlated with cortisol levels (36).

In a previous study, we demonstrated that a 500-fold increase in serum leptin levels did not modify the day–night cortisol rhythm in fasted, pubertal male monkeys (37). In that study, however, we could not exclude a dual effect of leptin, increasing the hypothalamic CRH release (38) and inhibiting the adrenal cortisol release (21, 22).

In the present study we show that leptin affects neither the cortisol response to ACTH in male macaques *in vivo*, nor the *in vitro* response of human adrenal cells to FSK, suggesting that leptin does not regulate acute cortisol secretion in non-human primates.

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