Glucocorticoid-Induced Changes in the Quantity and Secretory Capacity of Individual Rat Somatotropes

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ABSTRACT
Chronic glucocorticoid treatment is complicated by growth failure. The study presented here was designed to investigate the effect of cortisone on growth hormone (GH) secretion by individual pituitary cells in young male rats. Beginning at 37 days of age, animals were injected sc with cortisone acetate (CORT; 5.0 mg/rat per day) or the same volume of saline (SAL) for 8 days. At 45 days of age, the body weights of the CORT animals (134.5 ± 5.5 g) were significantly less (P < 0.0005) than those of SAL controls (179.3 ± 4.2 g). The secretory capacity of dispersed pituitary cells was assessed by the reverse hemolytic plaque assay. Cells were exposed to six concentrations of GH-releasing hormone (GHRH) ranging from 0.01 to 3.0 nM. CORT treatment significantly decreased the absolute number of somatotropes per pituitary gland (CORT, 1.23 ± 0.03 × 10⁶; SAL, 1.57 ± 0.09 × 10⁶; P = 0.025). Conversely, the mean plaque areas were significantly greater for CORT animals at all concentrations of GHRH tested, indicating that the amount of GH secreted by individual somatotropes was significantly increased by CORT. It was concluded that the paradoxical increase in the in vitro GHRH responsiveness, which is commonly observed after glucocorticoid treatment, was due to an increase in the capacity of fewer individual somatotropes to secrete GH.

Key Words: Growth hormone, growth hormone–releasing hormone, reverse hemolytic plaque assay

Glucocorticoid therapy is commonly used to treat kidney disease (1) and bronchial asthma (2). Treatment is complicated, however, by the growth failure that has been found to occur in both humans (1–3) and laboratory animals (4). Studies on the mechanism of growth failure have shown that glucocorticoids significantly influence the in vitro (5) and in vivo (6.7) secretion of growth hormone (GH), the numbers of GH-releasing hormone (GHRH) receptor binding sites (8), serum concentrations of insulin-like growth factors (IGF) (9), and somatostatin production (10). In a recent in vitro perifusion study of dispersed rat pituitary cells, we found that GH secretory rates in response to a range of GHRH concentrations were significantly elevated after in vivo injections of cortisone (CORT) (11). In view of these findings, this study was designed to use the reverse hemolytic plaque assay to investigate potential changes in the quantity and GH secretory capacity of individual pituitary somatotropes induced by cortisone treatment.

MATERIALS AND METHODS

Animals
Male Long-Evans rats were obtained at 25 days of age (Charles River Laboratories, Wilmington, MA) and were maintained in an environmentally controlled animal facility (12 h of light, 12 h of darkness), with the temperature between 21 and 23°C. Animals were fed standard rodent laboratory chow (Purina, St. Louis, MO) in a powdered form. Both food and water were available ad libitum. All animal procedures were conducted with prior approval by the Virginia Commonwealth University Institutional Animal Care and Use Committee (Protocol Number 8910-1123).

Experimental Design
Animals were weighed at 2- to 3-day intervals. Treatment with cortisone was begun at 37 days of age. Cortisone acetate (CORTONE: Merck, Sharp & Dohme, West Point, PA) was suspended in physiologic saline at a concentration of 50 mg/mL. Each animal received either CORT at a dose of 5.0 mg/day or saline (SAL) by sc injection. The treatment was continued for a period of 8 days, and pituitary tissue was collected on the morning after the completion of treatment (45 days).
Reverse Hemolytic Plaque Assay

The release of GH in response to GHRH by individual pituitary cells was documented by the reverse hemolytic plaque assay technique as previously described (12). Three rats provided pituitary tissue for each of the two experimental groups, and four repetitions of the experiments were performed on separate days with a total of 24 rats. On each experimental day, pituitary glands were collected under ether anesthesia after rapid decapitation. The posterior pituitary (neurohypophysis) was dissected free, and the anterior pituitary (adenohypophysis) was diced in minimal essential medium [SMEM; Gibco Laboratories, Grand Island, NY]. The fragments were transferred to 10 mL of SMEM containing 0.25% (wt/vol) trypsin ( Worthington Biochemical Corp., Freehold, NJ) and were placed in Bellco spinner flasks (Bellco Glass Co., Vineland, NJ) that had been silicon coated (Sigmacoat; Sigma Chemical Co., St. Louis, MO) to prevent the fragments and cells from sticking to the glass. The atmosphere of the flask was 95% oxygen and 5% carbon dioxide. Incubation and stirring were maintained for 1 h, after which the fragments were collected by centrifugation and transferred to SMEM containing 1% BSA (Sigma) to neutralize the trypsin. Cells were dispensed by gentle trituration with a 1.0-mL pipette (Pipetman; Rainin Instrument Co., Woburn, MA). An aliquot of cells was taken for determination of the numbers of cells by trypan blue dye exclusion test with a standard hemocytometer. The number of cells per pituitary gland was calculated as the total number of cells divided by the number of pituitary glands used (three in every case). The suspension was adjusted to a density of 4 × 10^5 cells/mL with Dulbecco’s modified Eagle’s medium (DMEM; Gibco). The pituitary cell suspension (0.5 mL) was added to 0.5 mL of a 12% suspension of ox erythrocytes that had been previously coated with protein A (Staphylococcus aureus; cat. # P-8143; Sigma). This cell mixture was dispensed into a culture chamber similar to that described by Cunningham and Szenberg (13) constructed on a glass slide treated with poly-L-lysine (0.2 mg/mL; 20 min). After a 60-min incubation at 37°C to allow cell attachment, nonadherent cells in the chamber were washed free by the slow application of DMEM (with 0.1% BSA, 20 mM N-hydroxyethylpiperazine-N’-2-ethanesulfonic acid [HEPES], and antibiotics [10 U/mL of penicillin, 0.5 μg/mL of streptomycin, 187.5 ng/mL of amphotericin, and 5 μg/mL of gentamicin]). After a further 30-min incubation, DMEM containing monkey antihuman GH antiserum (14) at a 1:200 dilution, with or without GHRH, was added to each chamber. GHRH concentrations were 0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 nM. Further control slides received no GH antiserum. The chambers were then incubated for 90 min, after which the antibody and GHRH were washed out with DMEM, guinea pig complement at a 1:25 dilution was added into the chamber, and incubation continued for 30 min. The hemolytic plaques were formed during this period. The cells were fixed with 1% glutaraldehyde, soaked in distilled water overnight, stained with methyl green pyronin, and then permanently mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

Quantitation

The percentage of cells forming hemolytic plaques was determined under GHRH stimulation. Slides were scanned with a light microscope (magnification, ×400) until a minimum of 500 nucleated cells were counted. Polymorphonuclear leukocytes and lymphocytes were excluded on the basis of their characteristic morphology. A hemolytic plaque was defined as the presence of a clear zone of ox red blood cell hemolysis wider than the diameter of the pituitary cell that entirely surrounded the cell in question. The proportion of plaque-forming cells to the total number of cells was defined as the percentage of somatotropes. The absolute number of somatotropes per pituitary gland was calculated as the product of the number of cells per pituitary gland and the percentage of somatotropes. This was done for each individual repetition, and the data were subsequently expressed as a mean ± SE. In all cases, the viability of the cells was more than 95% at this stage of the preparation. There are no further viability tests performed on the basis of the rationale that only viable cells would be able to form plaques. All cells are quantitated in the end, however, because the staining technique identifies all cells and this total number of cells is what is used for the calculation of the percentage of cells that secretes growth hormone. In this respect, any decrease in viability during the process of the assay would result in a uniform decrease only in the number of GH-secreting cells. We assume that the viability of the cells, which starts out fairly high, does not significantly decrease over the course of the assay.

There is no real measure of the sensitivity of the reverse hemolytic plaque assay because, unlike an RIA, it does not measure the hormone itself. In RIA, a standard curve with purified hormone is used to indirectly quantitate hormone levels in samples in nanograms per milliliter. The reverse hemolytic plaque assay measures the relative size of plaques surrounding cells that, by the nature of the technique, are known to secrete GH. The amount of hormone secreted has been approximated, but no exact quantitation is possible. Significant change in plaque area, however, is reliable and valid evidence for a significant change in GH secretion. It has been estimated that the sensitivity of the reverse hemolytic
plaque assay is in the picogram range. Because we measure secretion by millions of cells in the nanogram range with perfusion of dispersed cells (11), it is reasonable to consider such sensitivity in analyzing single-cell secretion. To estimate the amount of GH secreted from each somatotrope, the hemolytic plaque area was measured by a VideoPlan image analysis system (Carl Zeiss, Kontron, Munich, Germany), as described previously by Ho et al. (15). A minimum of 100 plaque areas was measured for each slide. Subsequent analysis was performed with the arithmetic mean of these plaque areas, and differences between the groups were assessed by generalized multivariate analyses (16). Statistical differences in body weight were analyzed by the same methods, and differences in pituitary cell numbers were determined by t test.

RESULTS

Weight Gain

Changes in body weight for the CORT- and SAL-treated animals are shown in Figure 1. Before treatment, mean body weights for both groups were identical. A significant inhibition of the normal increase in body weight became apparent within 3 days (40 days: SAL, 145.2 ± 4.1 g; CORT, 126.9 ± 4.9 g; P < 0.01) after the initiation of single daily injections of CORT at 37 days of age. At 45 days, the body weights for the two groups were markedly different (SAL, 179.3 ± 4.2 g; CORT, 134.5 ± 5.5 g; P < 0.0005).

Numbers of Somatotropes

The viability of the pituitary cells after dispersion was at least 95% for both groups in all experiments. The total number of cells per pituitary gland, the percentage of somatotropes, and the absolute number of somatotropes per pituitary gland at 45 days are shown in Table 1. CORT induced a decrease in the total number of cells per pituitary gland (P < 0.05), as well as a decrease in the percentage of somatotropes (P < 0.005). As a result, the decrease in the absolute number of somatotropes per pituitary gland became apparent (P < 0.025).

Mean Plaque Area

CORT treatment caused a significant increase in mean plaque area at all concentrations of GHRH tested. The concentration-response curves for GH secretion by individual somatotropes in response to GHRH, as indicated by changes in mean plaque area, are shown in Figure 2. The response to GHRH was significantly linear for each group, when determined with respect to the log of the GHRH concentration (CORT, r² = 0.988, P < 0.0001; SAL, r² = 0.975, P < 0.0001).

![Figure 1. Inhibition of the normal increase in body weight by CORT treatment as compared with that by SAL controls. All values are expressed as mean ± SE. Eight days of CORT injections (5.0 mg/day; sc) began at 37 days of age, and pituitary tissue was collected at 45 days of age (45d). Growth retardation was significant after 3 days of treatment (P < 0.01), and body weight remained depressed for the duration of the experiment.](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C/P (×10⁶)</th>
<th>%SOM</th>
<th>SOM/P (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL treated</td>
<td>3.59 ± 0.19b</td>
<td>43.7 ± 0.5c</td>
<td>1.50 ± 0.09d</td>
</tr>
<tr>
<td>CORT treated</td>
<td>3.12 ± 0.06b</td>
<td>39.5 ± 0.4c</td>
<td>1.23 ± 0.03d</td>
</tr>
</tbody>
</table>

* The table shows the total numbers of cells per pituitary gland (C/P), percentages of somatotropes (%SOM), and absolute numbers of somatotropes per pituitary gland (SOM/P) after CORT or SAL treatment. The data are expressed as mean ± SE (N = 4).

** Significantly different at P < 0.05.

* Significantly different at P < 0.005.

** Significantly different at P < 0.025.

DISCUSSION

A number of studies have investigated the question as to why growth is inhibited by glucocorticoids, and the question remains somewhat controversial. With
thought to be increased by glucocorticoid treatment. In a recent study of dispersed pituitary cells by in vitro perfusion, we found that treatment of 37-day-old male rats with cortisone resulted in an increase in GH responsiveness to a range of GHRH concentrations (11). Those findings agree with those of previous studies showing that GHRH-stimulated GH secretion was increased by incubation with glucocorticoids in vitro and by prior in vivo treatment (5, 7, 21).

This study is the first to show that the numbers of somatotropes and their individual secretory capacity are changed by CORT treatment. The decrease in overall numbers of cells per pituitary gland in CORT-treated rats agrees with findings of a previous study by Kohler et al. (5) illustrating a decrease in the numbers of cultured pituitary cells after in vitro glucocorticoid treatment. Our data showing a specific decrease in the somatotrope population, coupled with the specific increase in mean GH plaque areas for individual somatotropes, demonstrate that the commonly observed increase in in vitro GH release after glucocorticoid treatment is the result of an increased secretion of GH by a smaller number of individual cells.

All values presented are the means of the four experiments. Part of the reason why the standard errors were so low in the concentration-response data in the plaque assays was that the numbers of measurements of plaque area were very large. We measured plaque areas for at least 100 GH-secreting cells on each slide. Each GHRH concentration for each animal was done on at least one slide, which amounted to at least 400 individual values of plaque area over the four replicates of the experiments. With respect to the total numbers of cells per pituitary gland, we counted the numbers of viable cells at least twice. On the percentages of somatotropes, we counted at least 500 total cells per slide with the percentages of somatotropes calculated relative to that total number. The limitations of the reverse hemolytic plaque assay are numerous, but none of them were of significant concern in this study in which the data were extremely consistent. These findings are likely to continue to be consistent in the hands of other researchers who might be interested in the phenomenon of apoptosis or growth and GH secretion, all of which seem to be significantly influenced by glucocorticoids.

GH-secreting cells are known to respond to any other peptide or nonpeptide stimulus that causes GH release. We have not tested the degree of efficacy of the GHRH preparation. We used the GHRH we have used in extensive in vitro and in vivo experiments. The required duration of exposure (90 min), however, has made other techniques such as perfusion of dispersed pituitary cells and RIA of GH much more useful for determining the relative releasing capacity.

**Figure 2.** Augmentation of the GH secretory capacity of individual somatotropes after CORT treatment. The responses to six concentrations of GHRH, ranging from 0.01 to 3.0 nM, are shown. The sizes of the GH plaques surrounding individual somatotropes (Plaque Area) are expressed as mean ± SE in square micrometers (μm² × 10⁵). Values for CORT-treated cells were significantly higher at all concentrations of GHRH tested (P = 0.0007 for all except 0.03 nM (P = 0.0035)). Identifications of the lines are the same as in Figure 1.

respect to GH secretion, in vivo experiments indicate that glucocorticoids inhibit the GH response to GHRH in both humans and intact animals. One investigation reported inhibition of GHRH-stimulated GH release by hypoadrenalinism and reversal after glucocorticoid replacement therapy (17). Most studies in humans, however, have shown that glucocorticoids exert a significant inhibitory effect on GH release (6, 18, 19). Likewise, the most recent work by Wehrenburg et al. (20) with laboratory animals shows that dexamethasone inhibits the in vivo release of GH in response to GHRH. Stimulation of hypothalamic somatostatin secretion was proposed as the underlying mechanism, because glucocorticoid inhibition was overridden by antibodies to somatostatin.

In contrast to the inhibition of GH secretion in vivo, in vitro GH responsiveness of pituitary tissue is
of other substances. Exposure times to GH-releasing stimuli in those cases are 2.5 min or less, making them much more precise than the reverse hemolytic plaque assay. Further refinements of the reverse hemolytic plaque assay will be necessary to make it feasible to have a precise measure of hormone secretion by individual cells.

We did not measure changes in the length of the rats as a function of treatment. The inhibition of overall growth was readily apparent, however, and previous studies have shown a significant decrease in body length (4,11). Those were the studies upon which we based our treatment regimen, so we assumed that the inhibition of overall growth would be equally effective, which certainly was the case for body weight. We did not measure pituitary weight but would expect to have seen a decrease that would correlate with the decrease in body weight. We did find a significant decrease in pituitary cell number (Table 1), which would also indicate a decrease in pituitary weight.

Glucocorticoids are known to increase GH secretion by a wide range of mechanisms. These include increases in pituitary GHRH receptor capacity (8) and increases in production by both GH gene transcription (22) and subsequent GH synthesis (5). The question now arises as to how this strong facilitation of GH production at the pituitary level coexists with the apparent inhibition of GH release in vivo. The glucocorticoid-induced increase in GH secretion could lead to a change in the hypothalamic mechanisms that regulate GH secretion. Previous studies have shown that GH feeds back on the hypothalamus to increase somatostatin synthesis and secretion (23) and to decrease GHRH output (24). In this respect, in vivo GH inhibition could be the result of hypothalamic somatostatin release in response to GH hypersecretion. Wehrenberg et al. (20) have described the probability of increased somatostatin secretion in response to glucocorticoid treatment, as mentioned above. Further studies will be necessary to determine if the potential increase in somatostatin is a primary effect of glucocorticoids or a secondary response to increased pituitary GH secretory capacity.

Glucocorticoids have also been found to affect other specific growth factors. Somatomedin activity in children was decreased by methylprednisolone, probably because of an increase in somatomedin inhibitors (25). Glucocorticoids have also been shown to inhibit the production of IGF-I mRNA in liver (26), as well as in bone (27). In view of the negative feedback of IGF-I on GH secretion (28), a decrease in IGF-I would correlate with our observed increase in GH secretory capacity. IGF-I is also known to stimulate hypothalamic somatostatin production (29). In this regard, lower IGF-I levels would not correlate with an increase in hypothalamic somatostatin secretion after glucocorticoids. This again points to the direct augmentation of hypothalamic somatostatin by glucocorticoids or by an increase in GH short-loop feedback. In either case, the inhibition of IGF-I by glucocorticoid treatment would be pivotal to the inhibition of body growth.

In summary, these findings demonstrate the glucocorticoid-induced inhibition of growth with paradoxical facilitation of pituitary GH secretory capacity. The augmentation of GH secretion in response to GHRH was not by an increase in the number of cells but by an increase in the capacity of fewer individual somatotropes to secrete GH. Other studies have shown that the underlying mechanism involves a spectrum of cellular activities, ranging from GH gene transcription to the numbers of GHRH receptors. The potential for increased pituitary GH feedback to secondarily affect hypothalamic somatostatin and GHRH secretion or for a direct influence of glucocorticoids on these mechanisms remains to be determined.

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