Short-Term Glucocorticoid Administration Decreases Both Hypothalamic and Pituitary Galanin Synthesis in the Adult Male Rat

Rebecca S. Brogan, Lisa K. Coney, William B. Wehrenberg, Gianlorenzo Beretta, and Andrea Giustina

Galanin (GAL) is a peptide that has been implicated in the regulation of the growth axis. It is generally accepted that GAL can increase serum growth hormone (GH) levels, although the underlying mechanism for this increase is unknown. It is well known that long-term glucocorticoid treatment alters in vivo GH secretion, since there is a decrease in serum GH in response to stimuli. It has previously been shown in our laboratory that administration of GAL can overcome the effects of glucocorticoid administration on GH secretion. The aim of the present study was to determine the effects of long-term glucocorticoid administration on the regulation of hypothalamic and pituitary GAL mRNA levels. Adult male rats were treated for 72 hours with the synthetic glucocorticoid dexamethasone ([DEX] 40 μg/kg/d intraperitoneal injections). RNase protection assays were performed on both the hypothalamus and pituitary for the presence of GAL mRNA. As expected, DEX significantly decreased somatic growth, as evidenced by a decrease (50%) in the weight gain of glucocorticoid-treated versus control animals. It was also demonstrated that in both the hypothalamus and pituitary, glucocorticoid treatment reduced the level of GAL mRNA (to 11% and 6.5%, respectively) compared with the control condition. We conclude that the decrease in GAL mRNA may lead to a decrease in GAL secretion, which in turn may be involved in the glucocorticoid-induced inhibition of GH secretion.

THE EFFECTS of elevated glucocorticoid levels on the growth axis are complex. An increase in the circulating level of glucocorticoids, regardless of whether the source is endogenous or exogenous, results in decreased somatic growth both in the laboratory rat and in humans.[1-3] However, if administered in vitro, glucocorticoids enhance pituitary growth hormone (GH) secretion from cultured cells[4,5] and increase the synthesis of GH mRNA.[6]

GH is primarily regulated by hormones found in the hypothalamus, the most important of which are GH-releasing hormone (GHRH), which is stimulatory, and somatostatin, which is inhibitory. However, it has also been recognized that the actions of somatostatin and GHRH are modulated by numerous other factors. Recently, it has been shown that the neuropeptide galanin (GAL) also has stimulatory effects on GH secretion.[7-9] The underlying mechanism for GAL regulation of the growth axis is unknown; however, it has been demonstrated by our laboratory that subcutaneous administration of GAL in both adult male and female rats results in an increase in somatostatin mRNA and a decrease in hypothalamic GAL mRNA and GH mRNA (Brogan et al, unpublished observations, 1998). These data suggest that GAL may work in conjunction with somatostatin to influence serum GH concentrations in the rat model.

Glucocorticoids are also thought to regulate GH secretion through a mechanism involving somatostatin. Several studies have suggested that the inhibitory effects of glucocorticoids are due to elevated somatostatin tone.[10,11] Using immunocytochemical and molecular analysis, our laboratory has demonstrated that somatostatin synthesis (mRNA levels) and storage in the median eminence are enhanced after long-term glucocorticoid treatment.[12]

However, it is unknown whether glucocorticoids have a role in regulating GAL synthesis and secretion. It may be hypothesized that with glucocorticoid treatment there is a decrease in the amount of GH secretion, which could provide feedback at the level of the hypothalamus to increase the amount of hypothalamic GAL synthesis and secretion. The decreased GH secretion observed with enhanced glucocorticoid tone may also provide feedback at the level of the pituitary to increase pituitary GAL synthesis and secretion. In fact, O'Halloran et al.[13] showed in adult male rats that after 28 days of long-term dexamethasone (DEX) treatment (1 mg/kg/d) GAL immunoreactivity decreased in the anterior pituitary to 50% of control values, while pituitary GAL mRNA increased.

However, we have previously shown that exogenous GAL administration can overcome the glucocorticoid-mediated GH inhibition in male rats.[14] The aims of the present study were to determine the effects of 72 hours of glucocorticoid treatment on the hypothalamic level of GAL mRNA, and to confirm and establish a time frame for the response of pituitary GAL mRNA to the same treatment.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (450 to 550 g; N = 12) were housed in a temperature- and humidity-controlled environment and exposed to a 15-hour light/10-hour dark cycle (lights on at 7:30 AM). Food and water were available ad libitum.

Control animals (n = 6) were treated daily (9:00 AM) for 4 days with intraperitoneal saline (0.5 mL). DEX-treated animals (n = 6) were also treated daily, including the day of death (12:00 to 3:00 PM), with the synthetic glucocorticoid DEX 40 μg/Kg/d (Decadron; Merck, Sharp and Dohme, West Point, PA). The dose was selected based on the ability to inhibit the GH response to GHRH in conscious rats[11] and to inhibit somatic growth.[15] The duration was selected based on the study by Seifert et al,[16] who showed changes in pituitary GHRH receptors within this time frame.

From the Departments of Biological Sciences and Health Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI; and Endocrine Section, Department of Internal Medicine, Brescia, Italy.

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Address reprint requests to Andrea Giustina, MD, Endocrine Section; II Internal Medicine, Spedali Civili di Brescia—P.le Spedali Civili 1, 25123 Brescia, Italy.

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Molecular Biology Procedures

Three hours after the last injection of DEX, the animals were killed by decapitation. The pituitary and hypothalamus, delineated anteriorly by the optic chiasm, posteriorly by the mammillary bodies, laterally by the sulci formed with the temporal lobes, and superiorly by a plane 3 mm dorsal to the ventral surface of the median eminence, were removed, pooled by treatment group to ensure that adequate amounts of total RNA were recovered, and stored at −80°C for 24 to 48 hours prior to RNA extraction. Total RNA was extracted by the method of Chomczynski and Sacchi[17] and quantified using a spectrophotometer at absorbance 260 and 280 nm. One microgram of RNA was size-fractionated on a 1.2% agarose gel containing 2.2 mol/L formaldehyde,[18] Further methodological details are as previously described.[19] Internal standards such as α-actin were not used as an internal control, because endocrine changes are known to modify their levels.[20,21] We used a clone for the 18S subunit for ribosomal RNA for a loading control. Typically, this varies between 8% and 15%.

For RNase protection assays, a radioactive antisense riboprobe was produced from a cDNA for GAL (supplied by Dr M. Vrontakis, University of Manitoba, Winnipeg, Canada). The clone was linearized with *Hin*dIII, and the cDNA was transcribed with an in vitro transcription kit (Ambion, Houston, TX). The riboprobe was hybridized overnight at 42°C with 5 μg total RNA from either control or DEX-treated animals. The RNA:RNA probe hybrids were digested with RNase One (Promega, Madison, WI) for 1 hour at 30°C. They were incubated with proteinase K for 15 minutes at 37°C, phenol/chloroform-extracted, precipitated, and centrifuged.[22] After centrifugation, the hybrid pellets were resuspended, denatured in a 100°C water bath, resolved on 8% acrylamide denaturing gel, and autoradiographed for 15 hours at −80°C. Control experiments, such as undigested riboprobe and riboprobe digested with RNase One, were also performed.

Densitometry

Densitometry was performed using the Bioscan Optimas Digital Imaging system, version 3.01 (Bioscan, Edmonds, WA). Autoradiograms were projected onto a Sony Trinitron Color Video Monitor (Sony Communications Products, Teaneck, NJ) and then adjusted for optimal contrast and brightness. Background illumination corrections were made by removing reflected light from the radioactive mRNA band image. Average optical density levels for the total area were recorded using a computer subroutine (designed by Howard W. Bielich, Computer Imaging Applications, Madison, WI) that integrates the mean grayscale values for the areas demonstrating a band corresponding to the protected probe. Densitometric results for RNase protection assays are reported as a percent of the value obtained from control rats (control value, 100%).

Statistics

Data for the weight gain of control and DEX-treated animals were analyzed using a *t* test for independent samples. Weight data are presented as the mean ± SEM, and significance was assigned at a *P* level less than .05. Statistical analysis was not performed for mRNA levels, as the tissues were pooled, allowing recovery of adequate amounts of RNA (producing n = 1).

RESULTS

Long-term glucocorticoid treatment is known to inhibit somatic growth both in humans and in laboratory animals. In the present study, DEX treatment significantly reduced somatic growth as measured by weight gain in adult male rats (Fig 1). During the 72-hour treatment period, control rats gained a mean of 22 ± 2 g, while DEX-treated animals gained only 11 ± 4 g.

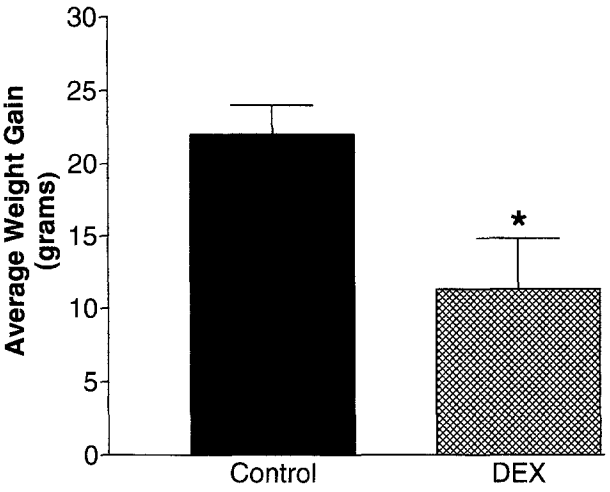

Fig 1. Mean weight gain of control and DEX-treated animals. The rats were treated for 72 hours with an intraperitoneal injection of either saline or 40 μg/kg/d DEX. Weight was measured before the injection on the first and last days of treatment. Data were collected by subtracting the weight on the first day from the recorded weight 72 hours later, and were analyzed using a *t* test for independent samples. *$P < .05$.

Figure 2 illustrates a RNase protection assay for GAL mRNA levels in control and DEX-treated hypothalamic and pituitary tissues. In lane 6, the cDNA for GAL produced an antisense riboprobe of approximately 800 nucleotides in length. Figure 2 also shows that the probe was completely digested with RNase One (probe +, lane 5).

DEX treatment reduced the level of pituitary GAL mRNA to 6.5% of the concentrated tissue. It also reduced the hypothalamic GAL mRNA level to 11% of the control tissue. The RNase protection assay is shown in Fig 2, and a summary graph is presented in Fig 3. The pooling of hypothalamic and pituitary samples did not allow statistical analysis of these results. However, the dramatic differences between control and treated tissues make it highly unlikely that the observed changes could be due to factors other than DEX treatment. The strength of the experiment is also increased by the fact that control tissues were obtained in animals studied in the same experimental conditions as the DEX-treated animals, randomly allocated to receive saline instead of DEX.

DISCUSSION

GAL has been shown to regulate anterior pituitary function, and specifically, it regulates GH secretion. Subcutaneous GAL administration increases the serum GH level and somatostatin mRNA and simultaneously decreases GH mRNA and hypothalamic GAL mRNA (unpublished observation). Long-term glucocorticoid treatment also influences the growth axis by decreasing somatic growth in both laboratory animals and humans. Administration of synthetic glucocorticoids such as DEX increases GH mRNA and somatostatin mRNA while at the same time decreasing GHRH mRNA and serum GH. Exogenous GAL administration has been demonstrated to block the glucocorticoid-mediated GH inhibition in male rats.[14] In fact, our data demonstrated that a 7-day high-dose treatment of glucocor-

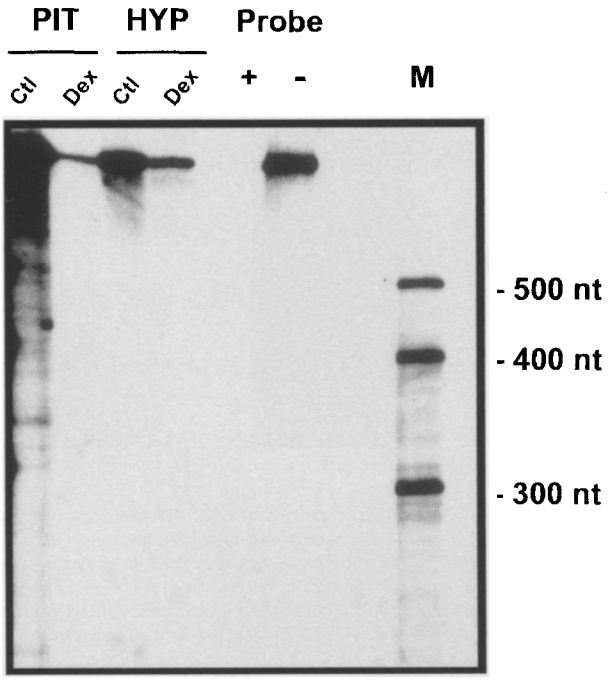

Fig 2. Scanned image of the autoradiogram for RNase protection assay. The riboprobe was the full-length cDNA for GAL. Lane 1, control pituitary; lane 2, DEX-treated pituitary; lane 3, control hypothalamus; lane 4, DEX-treated hypothalamus; lane 5, GAL riboprobe digested with RNase One; lane 6, GAL riboprobe without RNase One showing approximate size of 800 nucleotides (nt); lane 7, RNA size markers (Century Markers; Ambion, Houston, TX). All tissues were pooled; n = 6.

ticoids does not impair the GH response to GAL. Most interestingly, GAL reversed the blunted GH response to GHRH in glucocorticoid-treated rats. Administration of pharmacologic doses of glucocorticoids is thought to decrease the GH response to GHRH through an increase in hypothalamic somatostatin

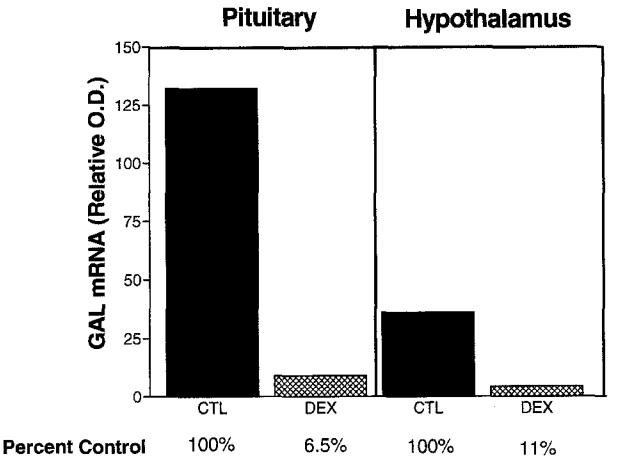

Fig 3. GAL mRNA data in control and DEX-treated animals. Data were collected using computer-assisted densitometry. Data are presented in relative optical density (O.D.) units. GAl levels in DEX-treated tissues are reported as a percent of the control tissues (control tissues, 100%).

tone. The fact that GAL stimulates GH secretion in a similar fashion in both long-term vehicle- and DEX-treated rats suggests that the mechanism of the GH-releasing activity of GAL in the rat may involve, in part, a decrease in somatostatin release by the hypothalamus.[23] It is not known if administration of glucocorticoids may affect GAL synthesis, and if these effects may be involved in the glucocorticoid-mediated inhibition of GH secretion. The aim of the present study was to determine the effects of short-term pharmacologic glucocorticoid administration on hypothalamic and pituitary GAL mRNA levels.

The mechanism of GAL regulation, at both the physiologic and molecular levels is unclear. It has been shown that GAL gene expression can be influenced by steroid hormones such as estrogen and corticosterone.[24]

The mechanism underlying this type of regulation may involve the anatomic location of steroid hormone receptors and GAL-producing neurons. De Leon et al[25] used double immunofluorescence techniques to show that the glucocorticoid receptor can be localized to neurons in the trigeminal ganglia and spinal cord that contain GAL immunoreactivity. This study provides an anatomic basis for the regulation of GAL-producing neurons by steroid hormones such as glucocorticoids.

In a different study, Torsello et al[26] used Northern blot analysis and in situ hybridization to demonstrate that steroid hormones could indeed regulate GAL gene expression and that the regulation was steroid- and tissue-specific. This group showed that in the adult male rat, GAL mRNA in tissues such as the adrenal gland and prostate is not influenced by either synthetic glucocorticoids or estrogens. They also showed that in tissues such as the testis, thymus, seminal vesicles, colon, and medial basal hypothalamus, GAL mRNA is indeed detectable but is not influenced by any steroids. Conversely, it was demonstrated that in the vas deferens and epididymis, GAL mRNA levels were increased after DEX treatment, but only in the vas deferens was GAL mRNA increased after estrogen treatment. These tissue-specific differences may be due to the presence or absence of accessory factors such as GAL-associated protein.

In the present study, we found that both hypothalamic and pituitary GAL mRNAs are decreased after 72 hours of daily injections of 40 μg/kg/d DEX. The relevance of this observation to the pathophysiology of glucocorticoid regulation of GH secretion is strengthened by the proposition that this is the period that allows deep glucocorticoid inhibition of serum GH levels in the rat.[11,14] To our knowledge, this is the first reported study of the effects of DEX treatment on the level of hypothalamic GAL gene expression. However, it has been reported by O'Halloran et al[13] that pituitary GAL mRNA increased after 28 days of 1-mg/kg/d DEX treatment in adult male rats. A possible explanation for this discrepancy is that the regulation of GAL gene expression may be sensitive to the dosage of steroid and the length of time it is used. In fact, Torsello et al[26] showed a dose-dependent increase in vas deferens GAL mRNA after 6 hours following a single DEX injection. The dose-dependent increase was observed at a dose of 0.1 to 100 μg DEX, although for dosages in the milligram range, vas deferens GAL mRNA did not increase further. Torsello et al also demonstrated that for a single injection of 1.0 μg/kg, the maximal effect was reached

between 6 and 9 hours later. These data may suggest that the observation by O'Halloran et al[13] represents the plateau of the pituitary GAL mRNA response to such a large and extended dosage of DEX.

In conclusion, the results of this study confirm and extend the data for the effects of short-term pharmacologic glucocorticoid administration on the growth axis. We have verified that DEX administration does inhibit somatic growth, as evidenced by the significant difference in weight gain in control versus DEX-treated animals. We also demonstrated that 72 hours of 40 μg/kg/d DEX decreases hypothalamic and pituitary GAL mRNA levels. We hypothesize that the reduction in GAL mRNA leads to a decrease in GAL secretion, which in turn could be involved in the increased hypothalamic somatostatin tone hypothesized as the mechanism of the ability of glucocorticoids to inhibit GH secretion[27] (Fig 4). However, it remains to be demonstrated that the described decrease in GAL mRNA is causally related to the inhibition of GH secretion previously shown to be induced in vivo by corticosteroids.[23] Future studies with long-term GAL administration should also address the potential involvement of decreased GAL synthesis in the growth retardation caused by long-term glucocorticoid administration.[10]

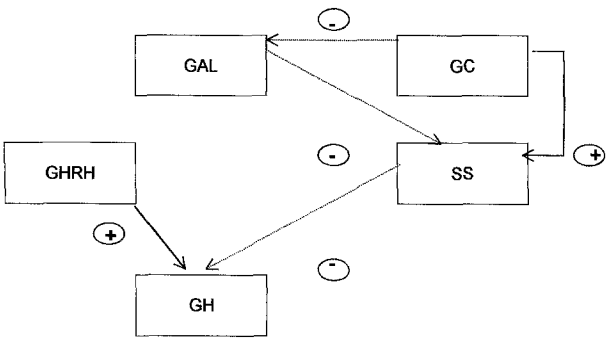

Fig 4. Proposed schematic representation of the GAL-glucocorticoid (GC) interaction with the somatostatin (SS)/GHRH/GH axis. GAL stimulates GH via decreased SS tone. GC-mediated GH inhibition occurs via increased SS tone. The GC-induced decrease in GAL synthesis may mediate the GC-induced increase in SS and consequent decrease in GH. Solid arrow, stimulation; broken arrow, inhibition.

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