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Regulation of Chicken Embryonic Growth Hormone Secretion by Corticosterone and Triiodothyronine

Evidence for a Negative Synergistic Response

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We reported that growth hormone (GH)-secreting cells differentiated by d 16 of chick embryonic development and that these somatotrophs were responsive to GH-releasing hormone and thyrotropin-releasing hormone. The present experiments evaluated effects of corticosterone and triiodothyronine (T_3) on embryonic GH secretion. Anterior pituitary cells from embryonic day (e) 16, e18, and e20 were subjected to reverse hemolytic plaque assays (RHPAs) for GH in the absence or presence of corticosterone or T_3. Corticosterone increased GH secretion from embryonic somatotrophs, an effect particularly evident on e16 and e18. T_3 decreased GH secretion on e16, while no effect of T_3 was significant on e18 or e20. Next, pituitary cells were subjected to RHPAs with T_3 and corticosterone alone or in combination. Combined treatment with these hormones suppressed GH secretion from e16, e18, and e20 somatotrophs to levels below those found under basal conditions. We conclude that corticosterone can stimulate GH secretion in vitro at all embryonic ages tested. Furthermore, T_3 can suppress basal GH secretion on e16, and the combination of T_3 and corticosterone can suppress GH secretion at all ages. These findings indicate that GH secretion during the end of chicken embryonic development may be regulated by the interactions of endogenous glucocorticoids and thyroid hormones that increase prior to hatching.

Key Words: Somatotroph; thyroid; pituitary; glucocorticoid; embryo; development.

Introduction

We have shown previously that somatotrophs differentiate between embryonic days (e) 14 and e16 in the chicken *(1)*. Others found that growth hormone (GH) was detected in plasma by e17 *(2)*, and GH mRNA was present by d 18 of chick embryonic development *(3)*. We have also shown that secretion of GH by embryonic somatotrophs is regulated by GH-releasing hormone (GHRH), thyrotropin-releasing hormone (TRH), somatostatin, and insulin-like growth factor-1 *(4–6)*. Compared to somatotrophs, thyrotrophs appear earlier in embryonic development, on d 6.5 *(7)*, and thyroid binding sites for thyroid-stimulating hormone are present as early as d 5.5 *(8)*. However, functionality of the thyroid axis occurs between d 10.5 and 12.5 of development *(9–13)*. Serum thyroxine (T_4) and triiodothyronine (T_3) concentrations increase by the end of embryonic development prior to hatching *(14)*. Adrenal glucocorticoid production and serum concentrations of glucocorticoids increase by e15 *(15,16)*. Thus, thyroid hormones and glucocorticoids are present in the systemic circulation from the time of somatotroph differentiation, and their presence raises the possibility for effects on embryonic GH secretion.

Studies have been conducted to determine the effects of T_3 and glucocorticoids alone or in combination on GH release in mammals *(17–19)*. The results suggest that these treatments act synergistically to increase the amount of GH released. By contrast, administration of thyroid hormones to posthatch chickens decreases GH synthesis and secretion *(20–25)*. Similarly, treatment of chicken pituitary glands with T_3 in vitro decreases GHRH- or TRH-stimulated, but not basal, GH release *(20,23,26)*. However, little is known about the contribution of thyroid hormones to the regulation of GH secretion during chick embryonic development. As for glucocorticoids, we have shown that corticosterone can induce GH cell differentiation in cultures of e12 chicken pituitary cells *(27,28)* and when administered directly to developing embryos on e11 *(29,30)*. By contrast, corticosterone is known to inhibit GH secretion after hatch *(31,32)*.

The purpose of the current study was to evaluate the effects of T_3 and corticosterone alone and in combination on secretion of GH from already differentiated somatotrophs during late embryonic development. Reverse hemolytic plaque assays (RHPAs) were used to assess the effects of these treatments on GH release from individual somatotrophs present on e16, e18, and e20.

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Results

Experiment 1: Effect of Corticosterone on GH Secretion

First, we examined the effect of corticosterone on GH secretion from e16, e18, and e20 chicken pituitary cells. Dissociated cells from each age were subjected to RHPAs in the absence and presence of corticosterone. Under basal conditions, eventually every somatotroph will form a plaque owing to constitutive release of GH. To detect significant stimulatory effects, incubation intervals must be used that are shorter than that required to reach maximal plaque formation under basal conditions. Likewise, longer incubation intervals must be used to detect inhibitory effects relative to basal plaque formation. Preliminary trials indicated that corticosterone acted to increase embryonic GH secretion. Based on this observation, cells were treated with corticosterone (10^{-12} to 10^{-8} *M* in 10-fold dilutions) for 6, 12, or 18 h of incubation, intervals shorter than that needed for all somatotrophs present to form plaques under basal conditions. Figure 1 presents the results from these trials. No significant differences were found after 6 h. After 12 h, plaque formation by e16 pituitary cells was not significantly different from basal for any concentration of corticosterone. Similarly, no concentration of corticosterone elicited a response above basal for e18 cells. However, the proportions of e20 cells that released GH in response to treatment with 10^{-11} to 10^{-9} *M* corticosterone were slightly increased from basal. After 18 h of incubation, treatment with 10^{-11} to 10^{-8} *M* corticosterone significantly increased GH plaque formation by e16 cells, relative to basal. A similar response was seen for e18 cells, in which all doses of corticosterone resulted in significant increases over basal. No response to any dose of corticosterone was observed for e20 cells at this time point, because essentially all somatotrophs were detected, even under basal conditions. Therefore, corticosterone increased GH plaque formation at one time point (12 or 18 h) for all ages tested.

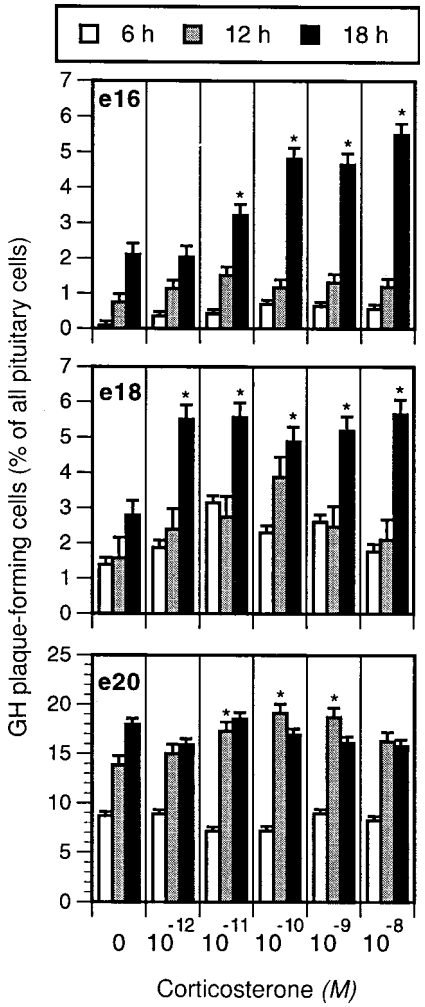

Fig. 1. Effect of corticosterone on GH secretion. Dispersed pituitary cells from e16, e18, and e20 were subjected to RHPAs. Cells were incubated for 6, 12, or 18 h with GH antibody (1:40) with or without corticosterone (10-fold dilutions from 10^{-12} to 10^{-8} *M*). Results represent percentages of GH plaque-forming cells detected at each time point and are the least square means ± SE from five independent trials. $*p < 0.05$ relative to basal.

Experiment 2: Effect of T_3 on GH Release

Next, cells from pituitaries of e16, e18, and e20 chick embryos were subjected to GH RHPAs in the presence of T_3. Preliminary trials were conducted that indicated an inhibitory effect of T_3 on embryonic GH secretion. To explore this inhibitory effect in detail, chambers containing e16, e18, or e20 pituitary cells were treated with 10-fold dilutions of T_3 (10^{-11} to 10^{-7} *M*) and incubated for 16 or 32 h. These intervals either approached or were greater than that required for all somatotrophs to form plaques under basal conditions, thus allowing detection of inhibitory effects. Figure 2 presents the results from this analysis. After 16 h of incubation, T_3 at 10^{-8} and 10^{-7} *M* significantly reduced the proportion of e16 cells that formed GH plaques. A similar response was seen after 32 hours of incubation, when T_3 at 10^{-10} to 10^{-7} *M* reduced the abundance of plaque-forming cells detected, relative to basal. No significant response to T_3 was detected on e18 or e20 at any time point or dose.

Experiment 3: Effect of Corticosterone and T_3 Alone or in Combination on GH Secretion

Finally, we examined the effect of corticosterone and T_3 alone or in combination on GH release. After determining the optimal dose for each agent in isolation, we performed preliminary experiments evaluating their combined effects in RHPAs. A complete dose-response study of corticosterone and T_3 in combination was not conducted, because an extensive analysis such as this would have required a minimum of 36 treatment groups. Analyzing each of these in duplicate RHPA chambers at multiple time points in replicate trials could not be accomplished easily with any accuracy. For this reason, we chose the optimal concentrations of corticosterone and T_3 determined from experiments 1

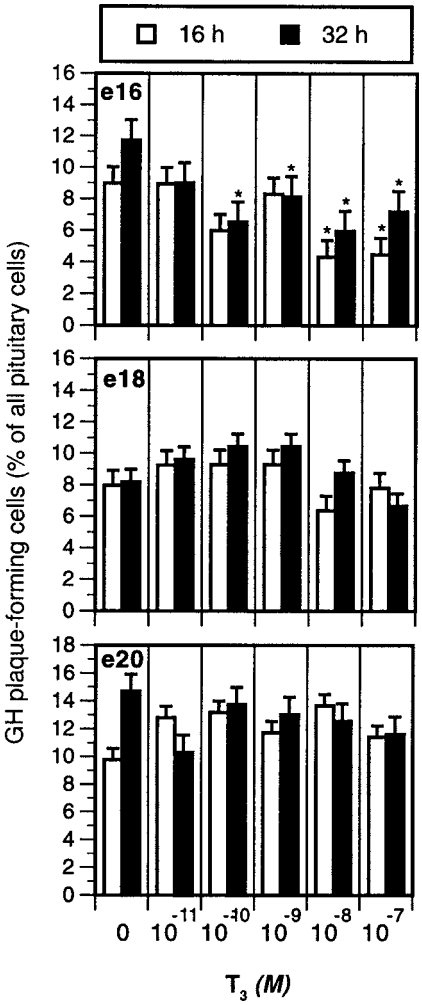

Fig. 2. Effect of T_3 on GH secretion. Isolated pituitary cells from e16, e18, and e20 were subjected to RHPAs for 16 or 32 h. Cells were incubated with GH antibody (1:40) with or without T_3 (10-fold dilutions from 10^{-11} to 10^{-7} *M*). Data presented are the least square means ± SE from four separate trials. $*p < 0.05$ relative to basal.

and 2. Analysis of the combined effects of corticosterone and T_3 was also problematic because both stimulatory and inhibitory effects were observed with the two agents in isolation. Initial trials, in which chambers were incubated for 6, 12, or 24 h, indicated that the combination of T_3 and corticosterone dramatically suppressed GH secretion, but this effect was observed only at 24 h. Thus, more and longer incubation intervals of 12, 18, 24, and 32 h were used in additional trials, in order to adequately evaluate this inhibitory effect. In all trials, pituitary cells from e16, e18, and e20 were subjected to RHPAs with T_3 (10^{-9} *M*) or corticosterone (10^{-9} *M*) alone or in combination. T_3 at 10^{-8} *M* was also evaluated with 10^{-9} *M* corticosterone in four of the trials. However, T_3 at 10^{-9} *M* in combination with corticosterone appeared more effective. For this reason, this concentration was selected for the remaining trials and for presentation. In total, three trials were run with time points of 6, 12, and 24 h and four trials with time points of 12, 18, 24, and 32 h. No significant effects were found at 6 or 12 h. Similar results were found at 18 and 24 h, but we present the results from 24 h (Fig. 3A) because more replicates were conducted at this time point (seven vs four). As in experiment 1, stimulatory effects of corticosterone were noted at the 18-h time point (data not shown). However, the effect of corticosterone alone was not significant after 24 h for all ages. This was likely owing to the formation of GH plaques by all somatotrophs under basal conditions at this extended interval. As found in experiment 2, T_3 in isolation significantly reduced the proportions of GH cells detected after 24 h on e16, but not on e18 or e20. By contrast, combined treatment with corticosterone and T_3 dramatically reduced the proportions of GH plaque-forming cells detected at 24 h to their lowest percentage for all ages. On e16 and e18, this proportion was significantly lower than that detected under basal conditions. All responses were diminished at 32 h, as they approached maximal plaque formation.

For this experiment, mean plaque area was also measured in order to evaluate relative amounts of GH secreted from individual somatotrophs. Mean plaque area for e16 cells treated with corticosterone and T_3 in combination was significantly smaller than mean plaque area of cells under basal conditions and those treated with corticosterone alone (Fig. 3B). On e18, cells treated with a combination of corticosterone and T_3 also produced plaques with a mean area significantly smaller than the mean plaque area of cells treated with corticosterone or T_3 alone. Similarly, on e20, cells treated with corticosterone and T_3 in combination produced plaques with average areas significantly smaller than those for cells under basal conditions and cells treated with either corticosterone or T_3 alone. Inspection of the frequency distributions for the sizes of plaques formed indicated that GH release from the majority of somatotrophs on each embryonic age was suppressed by combined corticosterone and T_3 treatment. Because combined treatment with corticosterone and T_3 reduced both the proportion of pituitary cells that formed detectable GH plaques and the mean area of the GH plaques formed, neither analysis alone reflects the whole extent of the treatment effects.

To provide an index of the cumulative treatment effects at 24 h, the product of plaque percentage and mean plaque area (area of GH plaques formed/100 pituitary cells) is presented in Fig. 3C. While a T_3 treatment effect was found for both plaque area and percentage on e16, no other significant effects of T_3 or corticosterone in isolation were noted at 24 h for any age. This was likely owing to the limitations inherent in the RHPA, wherein plaque formation is limited to one plaque per somatotroph once maximal plaque formation is reached. By contrast, combined treatment with T_3 and corticosterone reduced the product of the percentage of plaque-forming cells and the mean area of plaques formed to less than half of that found under basal conditions at all ages tested.

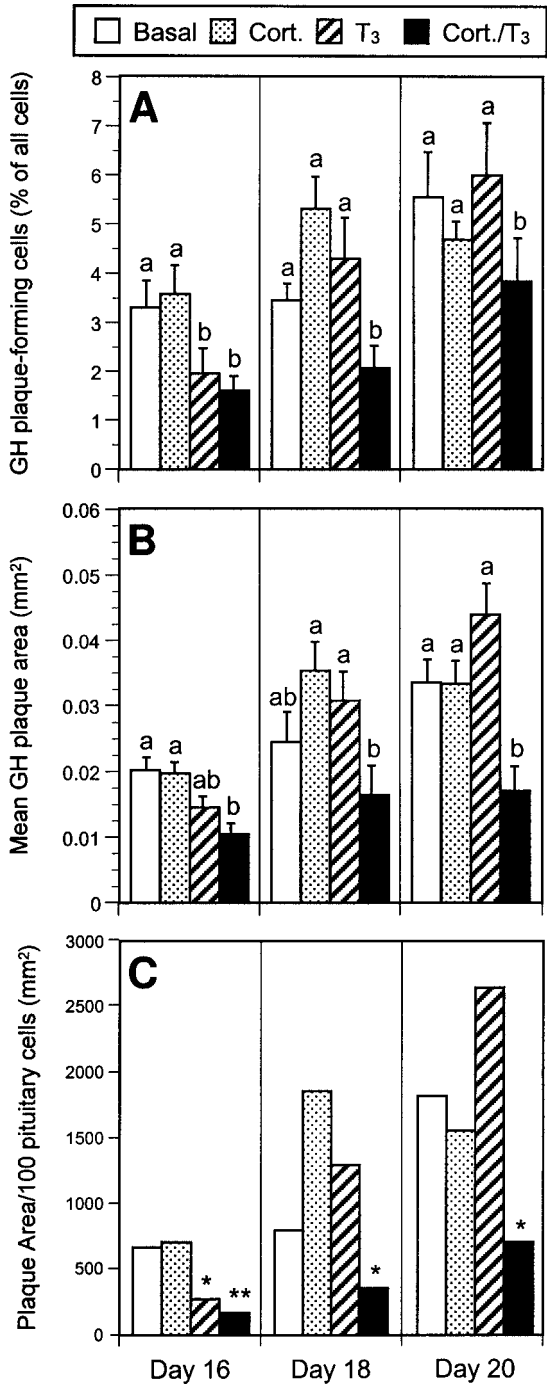

Fig. 3. Effect of coicosterone and T_3 in combination on the proportions of GH-releasing cells detected (**A**), the area of GH plaques formed (**B**), and the plaque area formed per 100 pituitary cells (**C**). Pituitary cells were isolated on e16, e18, and e20 and subjected to GH RHPAs for 24 h. Cells were incubated with GH antibody (1:40) with or without corticosterone or T_3 alone or in combination (10^{-9} *M* each). Data in (A) represent the percentages of GH plaque-forming cells and are the means ± SE from seven independent trials. The area of each plaque formed was measured, and results presented in (B) represent the mean area of the plaques formed (least square means ± SE from four independent trials). Values identified with different letters are significantly different from one another ($p < 0.05$). The average GH plaque area formed by 100 pituitary cells (C) was calculated by multiplying the proportions presented in (A) by the mean areas presented in (B).

Discussion

The effects of corticosterone and T_3 on GH secretion from chicken embryonic pituitary cells were evaluated. The formation of plaques in the RHPA was used to assess GH release. This approach was used because it is extremely sensitive, detecting GH release from individual embryonic somatotrophs. Corticosterone was found to stimulate GH secretion at all ages tested. However, the effect of corticosterone was more pronounced on e16 and e18 than on e20. This apparent decrease in responsiveness of somatotrophs on e20 may reflect a true decrease in the ability of glucocorticoids to increase GH secretion. In support of this possibility, others have noted that glucocorticoids decrease, rather than increase, GH secretion in chickens after hatch *(31,32)*. Thus, somatotrophs may be at a transition point in their response to corticosterone on e20. Alternatively, this apparent decrease in responsiveness on e20 may simply be an artifact owing to limitations inherent in the RHPA. Although the RHPA is extremely sensitive, once all somatotrophs present form GH plaques, a further stimulation by any agent is difficult, if not impossible, to detect. Thus, the apparent reduction in GH responsiveness to corticosterone on e20 may instead reflect an increase in basal GH release at that age. The basis for the diminished corticosterone response on e20 notwithstanding, our results indicate that glucocorticoids stimulate GH secretion during late embryonic development of the chicken. In addition to our current findings with somatotrophs present on e16, e18, and e20, we have also reported that corticosterone can induce the differentiation of GH cells in cultures of e12 pituitary cells in vitro *(27,28)*. The differences reported between embryonic and posthatch chickens suggest that the polarity of the GH response to glucocorticoids reverses after hatch, from induction of GH secretion in embryos to depression of GH release in posthatch chickens. By contrast, treatment with T_3 decreased GH release on e16 cells but had no significant effect on e18 or e20. Inhibition of GH secretion by T_3 on e16 is consistent with similar observations after hatch *(20–25)*, indicating a general inhibitory effect of T_3 on chicken GH secretion.

Perhaps the most intriguing finding of the present study was the inhibition of GH secretion by combined corticosterone and T_3 treatment. We found that combined treatment consistently suppressed the proportions of somatotrophs detected to basal levels or lower. This effect was found for all embryonic ages tested. Moreover, the area of the plaques formed in the presence of corticosterone and T_3 in combination was less than that of plaques formed under basal conditions or with corticosterone or T_3 alone for all embryonic ages. When the product of GH plaque percentage and

*Either the percentage of plaque formers or the mean area of plaques formed for that treatment were significantly less than under basal conditions ($p < 0.05$). **Both parameters were lower than basal.

mean plaque area was compared across treatments, we found that combined treatment with corticosterone and T_3 reduced this cumulative index of GH release to less than half of that under basal conditions for all ages tested. These observations indicate that combined corticosterone and T_3 treatment suppressed GH secretion below basal levels for all ages. Thus, while treatment with corticosterone alone increased GH secretion and treatment with T_3 inhibited GH release, but only on e16, combined treatment with both agents significantly inhibited GH secretion at all ages.

In the present study, we demonstrated that corticosterone and T_3 can modify GH secretion from somatotrophs present during later embryonic development of the chicken. We and others have shown that glucocorticoids and thyroid hormones are present in the systemic circulation from the onset of somatotroph differentiation and that their levels increase prior to hatch *(14–16)*. Corticosteroids increase from about e12, while T_4 and T_3 increase from about e15 and e20, respectively. Thus, stimulatory effects of glucocorticoids likely influence the somatotrophs present throughout late embryonic development. However, the inhibitory effects of thyroid hormones, alone or in combination with corticosterone, may be more prevalent just prior to hatching on e21, when levels of T_3 and T_4 are high. However, levels of GH in the circulation also rise around hatching *(2)*. This increase in circulating GH could indicate either that the inhibitory effects of T_3 in combination with corticosterone are not substantial in vivo or that the stimulatory effects of hypothalamic GHRH or TRH predominate around the age of hatching.

The present study illustrates the complex relationships between glucocorticoids and thyroid hormones on GH secretion. Each agent in isolation can affect chicken GH secretion in an age-dependent fashion. Moreover, the effect of combined corticosterone and T_3 treatment was not equal to the sum of the effects of treatment with each hormone alone. These findings indicate that glucocorticoids and thyroid hormones can act synergistically in the chicken to suppress GH secretion. Synergistic effects of glucocorticoids and thyroid hormones also have been reported in mammals *(17–19)*. In these previous studies, glucocorticoids and thyroid hormones acted synergistically to stimulate GH secretion. By contrast, we found, in the present study, that these classes of hormones can interact to suppress GH secretion during chicken embryonic development. This observation of a negative effect of combined corticosterone and T_3 treatment supports a fundamental difference in the regulation of GH secretion by glucocorticoids and thyroid hormones between birds and mammals.

Materials and Methods

Animals

Fertile eggs from Single Comb White Leghorn chickens (Hy-Line, Bryan, TX) were incubated at 37.5°C in humidified air, such that embryos at 16, 18, and 20 days of development would be available for experiments on the same day. In four to seven separate trials for each experiment, embryos were decapitated (three/age), and their anterior pituitaries were isolated with the aid of a dissecting microscope. The pituitaries were then dissociated by a combination of trypsin digestion and mechanical agitation as described previously *(1)*. Cell viability as assessed by the trypan blue dye exclusion method was >95%. The resulting cells were then subjected to RHPAs for GH. All cell culture media were supplemented with 50 U/mL of penicillin G, 50 µg/mL of streptomycin of sulfate (Life Technologies, Grand Island, NY), and 0.1% bovine serum albumin (Fraction V; Sigma, St. Louis, MO). Experiments were approved by the Animal Care and Use Committees at Texas A&M University and the University of Maryland.

Reverse Hemolytic Plaque Assays

RHPAs were performed as described previously *(1)*. Cells were mixed 1:1 with an 18% suspension of protein A–conjugated ovine erythrocytes, to provide a final pituitary cell concentration of 2×10^5 cells/mL. The cell suspension was infused into Cunningham chambers via capillary action and allowed to attach for 45 min in a humidified incubator (37.5°C; 5% CO_2, 95% air). Following the attachment period, unattached cells were rinsed out of the chambers using Dulbecco's modified Eagle's medium (DMEM). Corticosterone and T_3 treatments, which incorporated chicken GH antiserum (1:40 in DMEM; no. 4-011094), were then infused. Plaque formation using this antiserum has been shown to be specific for chicken GH release *(1)*. Chambers were incubated for specified amounts of time, after which guinea pig complement (1:40 in DMEM; Life Technologies) was infused. Chambers were then incubated an additional 45 minutes to allow for plaque formation. Finally, cells were fixed using 2% glutaraldehyde in saline and stored in phosphate-buffered saline containing 0.1% sodium azide until they were analyzed under a light microscope for plaque formation. Prior to analysis, anterior pituitary cells were stained using a 0.5% methyl green (Sigma) solution for 10 min and then destained with water. Pituitary cells were considered to be plaque formers if they were completely surrounded by a zone of hemolyzed erythrocytes at least one red blood cell thick in radius. The proportion of GH plaque-forming cells presented represents the number of plaque-forming cells observed divided by the total number of cells counted for a given slide (minimum of 200 cells/slide). The percentage of GH plaque formers from each chamber was then used as a single observation for statistical analysis.

Experiment 1: Effect of Corticosterone on GH Secretion

Pituitary cells from e16, e18, and e20 were subjected to RHPAs without (basal) or with corticosterone (Sigma) in 10-fold dilutions ranging from 10^{-12} to 10^{-8} *M* (two slides/

concentration/time point). Plaque assay chambers were incubated for 6, 12, or 18 h to allow for the detection of an increase in GH secretion by corticosterone, as indicated in preliminary experiments. This experiment was repeated in five independent trials. Slides were evaluated for percentages of GH plaque-forming cells.

Experiment 2: Effect of T_3 on GH Release

Pituitary cells from e16, e18, and e20 were tested without (basal) or with T_3 (Sigma) in 10-fold dilutions ranging from 10^{-11} to 10^{-7} *M* (two slides/concentration/time point). Chambers were incubated for 16 or 32 h to allow for the detection of an inhibition of GH secretion, as observed in preliminary experiments. This experiment was repeated in four independent trials. Slides were evaluated for percentages of GH plaque-forming cells.

Experiment 3: Effect of Corticosterone and T_3 Alone or in Combination on GH Secretion

Cells were incubated without (basal) or with corticosterone or T_3 alone or in combination, each at a concentration of 10^{-9} *M*. Chambers were incubated for 24 h to detect a decrease in GH secretion in response to the combined treatment (three chambers/treatment/time point). This experiment was repeated in seven independent trials, and slides were evaluated for percentages of GH plaque-forming cells and areas of plaques formed, to determine the relative amount of GH released per cell as described previously *(33)*. To measure the size of each plaque, slides were analyzed using a light microscope equipped with a calibrated optical reticle (Edmund Scientific, Barrington, NJ). Up to 100 plaques per slide were measured.

Statistical Analyses

Data were analyzed using the General Linear Model procedure of SAS, with statistically different groups identified by Duncan new multiple range test. The percentage of plaque-forming cells and mean area of plaques formed for each chamber were used as individual observations. Transformations were performed prior to analysis as needed, to ensure homogeneity of variance. Main effects included trial and treatment. For the dose response studies, each dose was considered a separate treatment. Data reported are the means ± SE of five, four, and seven separate trials, for experiments 1, 2, and 3, respectively. Differences were considered significant at $p < 0.05$.

Acknowledgments

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