

Thyroid Hormones Interact with Glucocorticoids to Affect Somatotroph Abundance in Chicken Embryonic Pituitary Cells *in Vitro*

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Our laboratory has reported that somatotroph differentiation occurs between d 14 and d 16 of chicken embryonic development and that corticosterone (CORT) can induce somatotroph differentiation at an earlier age *in vitro* and *in vivo*. The objective of the present study was to test for thyroid hormone-CORT interactions on somatotroph differentiation *in vitro*. Pituitary cells from d 11 chicken embryos were treated with CORT and thyroid hormones, and GH-producing somatotrophs were detected by reverse hemolytic plaque assays and immunocytochemistry. We found that thyroid hormones can act synergistically with CORT to further augment the abundance of somatotrophs *in vitro* but have little to no effect on their own. Both T₄ and T₃ could act synergistically with CORT to increase somatotroph abundance, but the effects of

T₃ were biphasic, inhibiting CORT actions at higher concentrations. The monodeiodination inhibitor iopanoic acid inhibited the synergistic effect of T₄ on CORT induction of GH cells *in vitro* but not the synergistic effect of CORT and T₃ or the effect of CORT alone. Furthermore, T₃ treatment overcame the iopanoic acid-induced reduction in the T₄-CORT effect. Our findings indicate that thyroid hormones act synergistically with CORT to further augment the abundance of somatotrophs *in vitro* and that conversion of T₄ to T₃ within the pituitary is involved in T₄ modulation of somatotroph abundance. Somatotroph differentiation during normal development may be regulated by complex interactions of hormones produced by the embryonic thyroid and adrenal glands. (*Endocrinology* 144: 3836–3841, 2003)

THE DEVELOPMENT OF the pituitary gland has been the subject of many studies aimed at elucidating the molecular mechanisms of generating diverse cell phenotypes from a common precursor within an organ (1, 2). The differentiation of pituitary cells is not autonomous but is controlled by intracellular and extracellular signals. The anterior pituitary-specific transcription factor Pit-1/GHF-1 is expressed in the thyrotroph, somatotroph, and lactotroph and is required for the proliferation and maintenance of these three cell types as well as the transcriptional activation of their corresponding trophic hormone genes (3). However, its expression pattern indicates that Pit-1 protein is not sufficient for somatotroph differentiation because GH is not expressed in the lactotroph or thyrotroph lineages, which also express Pit-1 protein. Thus, unknown cell-specific factors, in addition to Pit-1, exist that need to be identified to fully understand somatotroph differentiation during development.

Our laboratory is studying the mechanisms regulating differentiation of GH-secreting cells in the chicken anterior pituitary. During chicken embryonic development, which lasts 21 d, somatotrophs are rare before embryonic day (e) 12, and significant somatotroph differentiation occurs between e14 and e16 (4). GH-secreting cells do not differentiate spontaneously without an extrapituitary signal, and serum from e16 embryos but not e12 can induce somatotroph differentiation in culture (5). The adrenal glucocorticoid, corticosterone, is the active compound responsible for cell-differentiating activity of e16 chicken serum (6). Glucocorticoids

were found to induce somatotrophs in chicken embryos and fetal rats *in vitro* and *in vivo* (6–9). Furthermore, glucocorticoids were found to increase pituitary GH mRNA levels in rats (8), enhance GH mRNA stability in humans (10), and increase the level of GH gene expression and the number of cells that expressed GH mRNA in chickens (11).

Thyroid hormones play an important role in embryonic and neonatal development in mammals and birds. In humans, hypothyroidism severely impairs postnatal growth and reduces spontaneous nocturnal GH secretion (12, 13). T₃ was observed to increase GH mRNA accumulation in a cell line (GX) derived from a pituitary tumor of a boy with gigantism (14). In rats, thyroid hormones have been well documented to control GH synthesis. Thyroid hormones stimulated rat GH gene expression *in vivo* (15) and in several GH-producing pituitary cell lines (*e.g.* GH₁, GH₃, and GC) (16–18). In GC cells, T₃ was shown to rapidly stimulate GH gene transcription, and this effect was mediated by thyroid hormone receptors (19). However, thyroid hormones were found to exert their stimulatory action on fetal GH gene expression and GH cell abundance only in the presence of glucocorticoids (20). In chickens, the regulation of GH secretion by thyroid hormones is complex. The importance of T₃ to the normal growth process was demonstrated by the ability of exogenous T₃ to correct the growth deficit of hypophysectomized chickens (21). In contrast, administration of thyroid hormones to posthatch chickens decreased GH synthesis and secretion (22). However, nothing is known about the effects of thyroid hormones on somatotroph differentiation during chicken embryonic development.

Previous studies have evaluated the effects of combined

Abbreviations: e, Embryonic day; ICC, immunocytochemistry; IOP, iopanoic acid; RHPA, reverse hemolytic plaque assay.

thyroid hormone and glucocorticoid treatment on GH expression in the pituitary glands of fetal rats. Hemming *et al.* (23) found that T_3 , which was individually ineffective, acted synergistically with cortisol to increase somatotroph density in pituitary tissue from d 14 rat fetuses cultured for 7 d. Because GH immunostaining in histology sections of the cultured tissue was evaluated in this study, qualitative rather than quantitative results were obtained. Nogami *et al.* (20) found that adding dexamethasone to the drinking water of pregnant rats induced GH and GH mRNA accumulation in the d 18 fetal pituitary gland. Additional ip T_4 injections enhanced the effect of dexamethasone, whereas T_4 exhibited no effect when given alone. Because treatments were administered to the dams rather than directly to the fetuses in these studies, the potential involvement of a treatment-induced maternal mediator of these effects could not be eliminated. This group also examined the effect of glucocorticoids and T_3 on GH mRNA levels in cultured fetal pituitary glands (8). However, the abundance of somatotrophs was not evaluated in that study.

The purpose of the current study was to quantify direct effects of thyroid hormones and corticosterone on somatotroph abundance in cultures of chicken embryonic pituitary cells. Reverse hemolytic plaque assays (RHPA) and immunocytochemistry (ICC) were conducted to quantify the effects of these treatments on somatotroph abundance. The requirement for monodeiodination of T_4 in induction of somatotroph differentiation was also tested.

Materials and Methods

Animals and pituitary dispersions

All animals used in the present study were Avian X Avian broiler strain chicken embryos purchased from Allen's Hatchery (Seaford, DE). All procedures with chicken embryos were approved by the Institutional Animal Care and Use Committee on this campus. Unless stated otherwise, all cell culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY), and hormones and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All media were supplemented with 0.1% BSA, 100 U/ml penicillin G, and 100 μ g/ml streptomycin sulfate. Fertile eggs were placed in a humidified incubator (G.Q.F. Manufacturing, Savannah, GA) at 37.5 C. The typical length of incubation for chickens is 21 d. Embryos were removed on e11, and their anterior pituitary glands 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 (4–7). The viability of the cells was assessed by the trypan blue dye exclusion method and was consistently greater than 95%.

Extended cell cultures

Anterior pituitary cells were cultured according to the procedure described previously (4–6). Cells were plated (2.0×10^5 cells/well) in poly-L-lysine-coated 12-well tissue culture plates and allowed to attach for 45 min in 75 μ l DMEM. Wells were then filled with 2 ml serum-free medium (1:1 mixture of phenol red-free medium 199 and Ham's F-12, supplemented with 0.1% BSA, 5 μ g/ml human transferrin, 5 μ g/ml bovine insulin, 100 U/ml penicillin G, and 100 μ g/ml streptomycin sulfate) alone or medium containing different treatments. After culture for 3 or 6 d in a humidified incubator (37.5 C; 95% air-5% CO_2), cells were harvested for detection of GH-secreting cells by RHPA or GH-containing cells by ICC.

RHPAs

The assays were performed according to the protocol described in detail previously (4–6). Briefly, the harvested pituitary cells (1.0×10^5

/ml) were mixed with an equal volume of an 18% suspension of protein A-coated ovine erythrocytes and infused by capillary action into poly-L-lysine coated Cunningham chambers. After cells were allowed to attach for 45 min (37.5 C; 95% air-5% CO_2), chambers were rinsed with DMEM to remove unattached cells. DMEM containing GH antiserum (1:40) and human GHRH (10^{-7} M) was then added to the resulting monolayer of cells, and replicate chambers were incubated for 20 h (three chambers per treatment). Plaque formation was subsequently induced by a 45-min incubation with guinea pig complement (1:40, in DMEM). The cells were then fixed with 2% glutaraldehyde in 0.9% saline and stained with methyl green. Chambers were analyzed using a light microscope. Data shown are the percentage of all pituitary cells that formed plaques (*i.e.* secreted GH), with at least 200 pituitary cells counted/assay chamber.

ICC

The harvested pituitary cells were diluted in DMEM to a concentration of 1.0×10^4 cells/ml and were then attached to poly-L-lysine coated 24-well tissue culture plates during a 1-h incubation period (37.5 C; 95% air-5% CO_2). The plated cells were fixed with 3.7% formaldehyde in PBS for 20 min. Cells were permeabilized with 0.1% Tween 20/0.1% Triton X-100 (8 min), quenched with 0.3% H_2O_2 (5 min), blocked with 1% normal goat serum (15 min), and incubated overnight with rabbit antichick GH (1:4000 in PBS) using the antiserum described above for the RHPA. The cells were rinsed with PBS and incubated with biotinylated goat antirabbit IgG for 30 min. The cells were further processed using rabbit avidin biotin complex kits according to the directions supplied by the manufacturer (Vector Laboratories, Burlingame, CA). VIP reagent (Vector) was used as substrate for the peroxidase. GH-containing cells were identified using an inverted light microscope, and data shown are the percentage of the total pituitary cell population that contained GH.

Statistical analysis

The data reported are the mean \pm SEM from at least four completely separate experiments, with the number of replicate experiments provided in the legend to each figure. All data were analyzed by ANOVA using the mixed models procedure of SAS (SAS Institute, Cary, NC). Differences between treatments were tested using Tukey's Studentized range test and were considered significant at $P < 0.05$.

Results

Effect of corticosterone and T_3 alone or in combination on somatotroph abundance

We reported that corticosterone induces somatotroph differentiation within 2 d in cultures of chick embryo pituitary cells (6). We also showed that corticosterone and GHRH act synergistically after 3 d to further increase GH cell abundance (24). To extend our investigation of hormone interactions, pituitary cells from e11 chicks were cultured for 3 or 6 d with corticosterone (10^{-9} M) or T_3 (10^{-11} to 10^{-8} M) alone or in combination, and then cells were subjected to GH RHPA. Figure 1 presents the results from these trials. Corticosterone alone increased proportions of GH-secreting cells by 3 and 6 d to 5.5 ± 1.7 (mean \pm pooled SE of the mean) and $6.1 \pm 1.4\%$ vs. basal levels of $1.2 \pm 0.6\%$ and $0.3 \pm 0.2\%$, respectively. No significant effects were found for any concentration of T_3 alone. Therefore, T_3 alone had no effect on the abundance of somatotrophs in cultures of chick embryonic pituitary cells. However, low doses of T_3 in combination with corticosterone further increased the proportions of GH-secreting cells above that with corticosterone alone (to $10.3 \pm 1.8\%$ at 10^{-9} M T_3 after 3 d and $9.0 \pm 1.5\%$ at 10^{-10} M T_3 after 6 d). In contrast, high levels of T_3 inhibited the stimulatory effect of corticosterone on proportions of GH-secreting cells

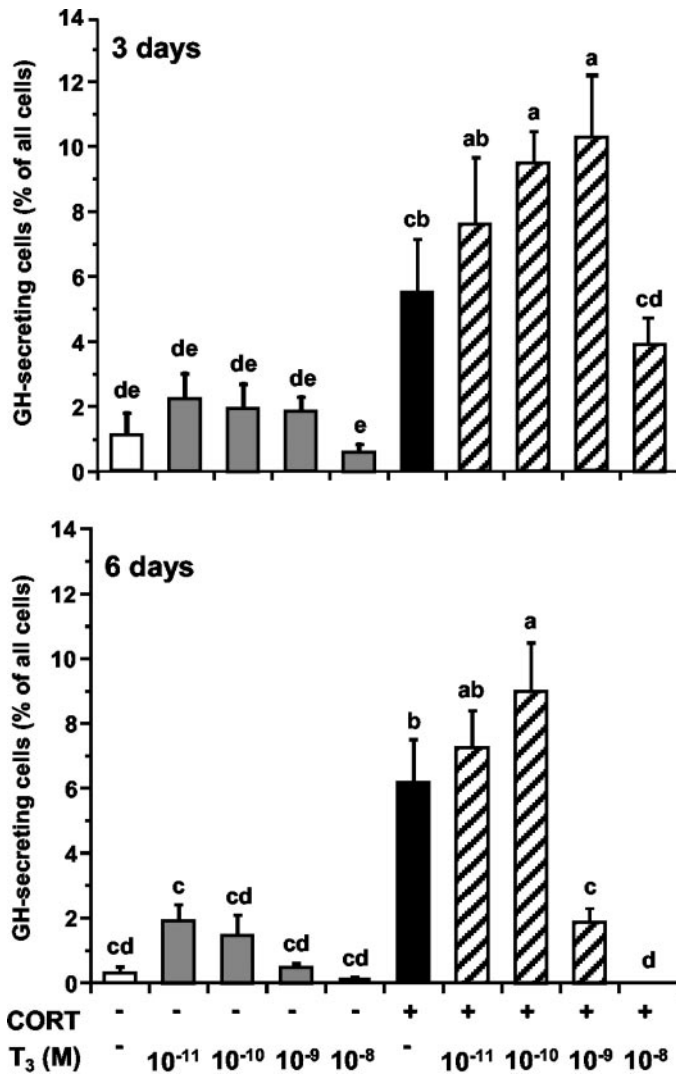


FIG. 1. The effect of corticosterone and T₃ on somatotroph abundance determined by RHPA. Pituitary cells from e11 chicks were cultured for 3 d (upper panel) or 6 d (lower panel) with corticosterone (10⁻⁹ M) or T₃ (10⁻¹¹ to 10⁻⁸ M) alone or in combination. Cells were harvested and subjected to RHPA for GH. Slides were incubated for 20 h in the presence of GHRH (10⁻⁷ M) to induce GH secretion. A minimum of 200 pituitary cells were counted in each of three replicate chambers for each treatment to determine the percentage of GH plaque-forming cells. These results are the means and SEM from four independent experiments. Values denoted by different letters are significantly different (*P* < 0.05).

(10⁻⁸ M T₃ for 3 d, 10⁻⁹ and 10⁻⁸ M T₃ for 6 d). Thus, T₃ regulates the induction of somatotroph differentiation by corticosterone in a biphasic, dose-dependent manner.

Effect of corticosterone and T₄ alone or in combination on somatotroph abundance

It is well established that circulating T₃ in birds and mammals is derived almost exclusively from the peripheral conversion of T₄ secreted by the thyroid gland. The next experiment was designed to examine the effect of T₄ on somatotroph abundance *in vitro*. E11 pituitary cells were treated with T₃ or T₄ (10⁻¹⁰ M, 10⁻⁸ M) with and without

corticosterone (10⁻⁹ M) for 3 d (Fig. 2). Similar to results with the RHPA above, corticosterone alone increased proportions of GH-containing cells to 8.9 ± 1.0% vs. basal levels of 0.5 ± 1.0%. T₃ at 10⁻¹⁰ M significantly increased the number of GH-containing cells induced by corticosterone after 3 d of culture (to 12.9 ± 1.1%), but T₃ at 10⁻⁸ M suppressed the effect of corticosterone (to 5.3 ± 1.1%). T₄ alone slightly increased the percentage of GH-containing cells. In contrast to T₃, T₄ significantly enhanced the effect of corticosterone on somatotroph abundance at both doses. T₄ at 10⁻⁹ M was chosen for the remaining experiments.

The next experiment was designed to determine whether the synergistic interactions with T₄ occurred across a wide concentration range for corticosterone. E11 pituitary cells were treated with corticosterone (10⁻¹¹ to 10⁻⁸ M) alone or in combination with T₄ (10⁻⁹ M) for 3 d. Treatment with 10⁻¹⁰ to 10⁻⁸ M corticosterone significantly increased GH cell abundance in e11 cells, relative to basal (Fig. 3). T₄ augmented the somatotroph abundance induced by all doses of corticosterone tested, indicating that the synergistic effect was not restricted to a limited range of corticosterone levels. However, corticosterone at 10⁻¹⁰ M in combination with T₄ appeared most enhanced (7.7 ± 1.3%; *P* < 0.01), relative to corticosterone alone (5.5 ± 1.3%). For this reason, this concentration was selected for the remaining trials.

Conversion of T₄ to T₃ is necessary for T₄ modulation of somatotroph abundance

The different dose-dependent effects of T₄ and T₃ likely reflect a difference in the mechanism of action of these thyroid hormones. The affinity for its receptor and the biological

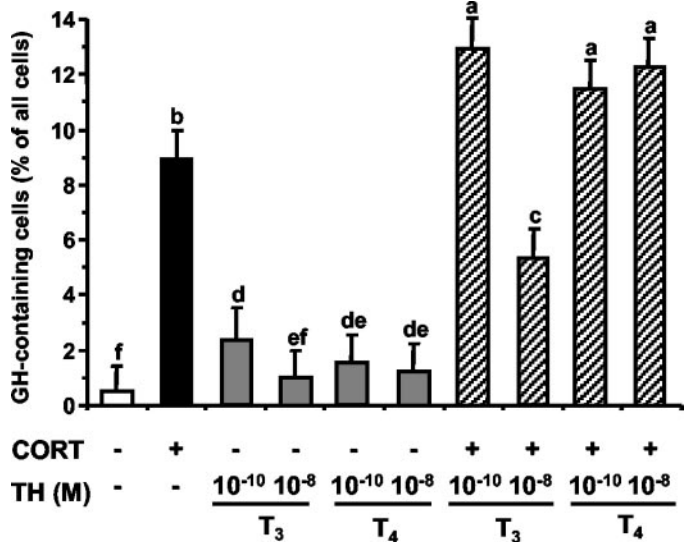


FIG. 2. The effect of corticosterone and T₃ or T₄ (TH) on somatotroph abundance determined by ICC. E11 pituitary cells were cultured for 3 d with corticosterone (10⁻⁹ M), T₃ (10⁻¹⁰ M, 10⁻⁸ M), or T₄ (10⁻¹⁰ M, 10⁻⁸ M) alone or in combination. Cells were harvested, and the percentage of pituitary cells that contained GH was determined using ICC. A minimum of 200 pituitary cells were counted in each of four replicate wells for each treatment. Data were analyzed by ANOVA, followed by Tukey's Studentized range test. Results shown are the means ± SEM of four independent experiments. Values denoted by different letters are significantly different (*P* < 0.05).

potency of T₃ is 10 times higher than that of T₄ in rat tissues and cultured cell lines derived from the rat and other species (25). It is well known that the type II 5'-deiodinase is responsible for conversion of T₄ to T₃ in the anterior pituitary of adult animals. Iopanoic acid (IOP) is both an inhibitor of all three types of deiodinases and a substrate for the pituitary type II 5'-deiodinase (26, 27). Furthermore, IOP has been shown to block T₄ to T₃ conversion in chick embryos from d

19 of incubation (28). So IOP was used to explore the basis for dose-dependent differences between T₄ and T₃. Based on preliminary experiments, we determined that 10⁻⁶ M IOP was not cytotoxic and did not affect corticosterone induction of somatotrophs. We next examined whether the conversion of T₄ to T₃ was necessary for the T₄ effect on somatotroph abundance. Cells from pituitaries of e11 chick embryos were cultured for 3 d in the presence of IOP (10⁻⁶ M), T₄ (10⁻⁹ M), T₃ (10⁻¹⁰ M), and corticosterone (10⁻¹⁰ M) alone and in combination. Figure 4 presents the results from these trials. Corticosterone increased the somatotroph abundance to 5.6 ± 0.9% vs. basal levels of 2.1 ± 0.9%. T₄ significantly enhanced the effect of corticosterone on somatotroph abundance (to 7.5 ± 0.8%). No significant effects were detected for IOP and T₃ alone, relative to basal. IOP did not affect the stimulatory effect of corticosterone, as we expected. In contrast, IOP blocked the slight increase in GH cell abundance induced by T₄ and inhibited the added effect of T₄ on corticosterone induction of GH cells (to 5.5 ± 0.9%). However, IOP did not suppress the combined effect of corticosterone and T₃. Interestingly, T₃ treatment overcame the IOP-induced reduction in the corticosterone/T₄ effect. Thus, conversion of T₄ to T₃ was necessary for T₄ modulation of somatotroph abundance.

Discussion

It has been demonstrated that somatotroph differentiation does not occur *in vitro* without an extrapituitary signal in rats and chickens. Previously we reported that e16 chicken serum could induce premature somatotroph differentiation, and adrenal corticosterone is the bioactive component of e16 serum (5, 6). We have found that corticosterone is capable of inducing GH cell differentiation *in vitro* and *in vivo* (6, 7). We

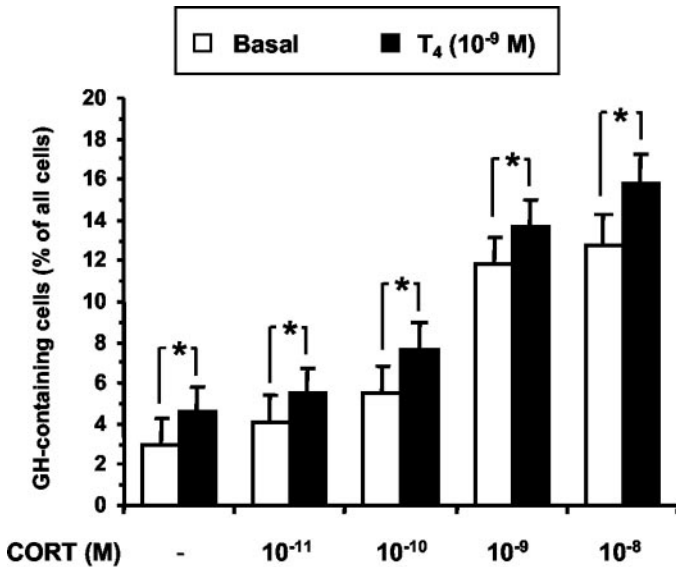


FIG. 3. Determination of the range of corticosterone doses for observing synergistic interactions with T₄. E11 pituitary cells were treated with corticosterone (10⁻¹¹ to 10⁻⁸ M) alone or in combination with T₄ (10⁻⁹ M) for 3 d. The percentage of GH cells was determined using ICC. Results shown are the means ± SEM of four independent experiments. Data were analyzed as in Fig. 2, with values denoted by an asterisk (*) being significantly different from each other (P < 0.05).

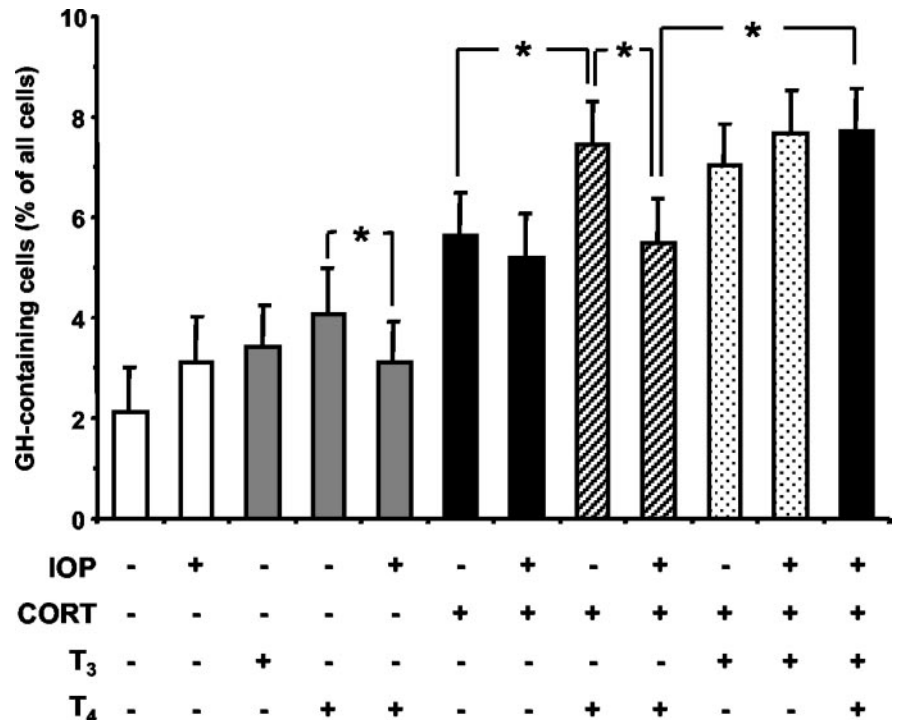


FIG. 4. Effect of monodeiodinase inhibition on thyroid hormone effects on somatotroph abundance. E11 pituitary cells were treated with corticosterone (10⁻¹⁰ M), T₄ (10⁻⁹ M), T₃ (10⁻¹⁰ M), or IOP (10⁻⁶ M) alone or in combination for 3 d. Cells were harvested, and the percentage of GH cells was determined using ICC. Results shown are the means ± SEM of four independent experiments. Data were analyzed as in Fig. 2, with values denoted by an asterisk (*) being significantly different from each other (P < 0.05).

have also found that serum concentrations of glucocorticoids increase by e14 (Liu, L., and T. E. Porter, unpublished data). In agreement with our previous work, we found in the present study that short- and long-term exposures to corticosterone increased somatotroph percentages among pituitary cells from e11, an age in which somatotrophs are normally rare.

Thyrotrophs have been identified early in embryonic development, on e6.5 in chickens (29), and functionality of the thyroid axis occurs between e10.5 and 12.5 of development (30). Serum T_4 and T_3 have been detected in chick embryos as early as e9.5 (31, 32). We measured plasma total T_4 and total T_3 from e9 through e14 (Liu, L., and T. E. Porter, unpublished data). Levels of both hormones increased between e11 and e13, with T_4 being twice as abundant as T_3 during this period. Levels of both total T_4 and total T_3 were at levels that were stimulatory to glucocorticoid effects in the present study ($\sim 10^{-10}$ to 10^{-9} M). Thus, thyroid hormones are present in the systemic circulation from the time of somatotroph differentiation. Thyroid hormones regulate GH gene expression through thyroid hormone receptors. Thyroid hormone receptors are the product of two genes, $TR\alpha$ and $TR\beta$. The chicken $TR\alpha$ gene produces a single $TR\alpha$ mRNA, which is translated into two proteins by the use of two alternative start codons, and the $TR\beta$ gene gives rise to two variant mRNAs, $TR\beta 0$ and $TR\beta 2$ (33, 34). The rat GH gene is thought to be a potential target for $TR\beta 2$ because this receptor has been reported to be highly expressed in rat somatotrophs (35), and it is the predominant TR in GH_3 cell nuclear extract that binds to the GH promoter response element *in vitro* (36). Analysis of expression of $TR\alpha$ and $TR\beta$ mRNA during chicken development demonstrated that $TR\alpha$ mRNA was ubiquitously present from early embryonic stages, whereas $TR\beta$ mRNA displayed restricted tissue specificity, occurring notably in brain, eye, lung, yolk sac, and kidney, and was subject to striking developmental control (37). However, no report has specifically examined expression in the pituitary.

Multiple studies suggest that thyroid hormones can activate rat GH synthesis and synergistically stimulate GH expression with glucocorticoids in rat pituitary cell lines (16–19). However, thyroid hormones appear to require glucocorticoids to regulate GH gene expression and somatotroph differentiation in fetal animals. Nogami *et al.* (8) found that T_3 increased GH gene expression in cultured fetal rat pituitary glands only in the presence of glucocorticoids. Similarly, although having no effect when given alone, T_4 injections into pregnant rats increased GH and GH mRNA accumulation in the corresponding fetal pituitary glands on d 17 or 18 of gestation, which were induced by dexamethasone in the drinking water (20). Hemming *et al.* (23) demonstrated that GH cells could be induced in cultured fetal rat pituitary primordia with cortisol. T_3 , which was individually ineffective, acted synergistically with cortisol to increase GH cell density in the sectioned tissue. These reports indicate that glucocorticoids and thyroid hormones can act synergistically to induce GH production in the fetal rat pituitary gland. Our present results using dissociated chicken embryonic pituitary cells in culture indicate that the synergistic actions of

glucocorticoids and thyroid hormones occur directly on the somatotroph precursor population to induce an increase in the absolute abundance of GH-producing cells.

To date there have been no reported studies on effects of thyroid hormones on somatotroph abundance during chicken embryonic development. In the present study, we demonstrated that T_3 or T_4 alone had minimal effects on the percentage of somatotrophs in e11 pituitary cell cultures using RHPA and ICC. Thus, thyroid hormones alone could induce GH cell abundance to a small extent. However, induction of somatotrophs by thyroid hormones alone was limited relative to the effects of combined corticosterone and thyroid hormone treatments. It was interesting to find that the corticosterone and T_3 interaction on somatotroph abundance was dose dependent. Similar to the results of Hemming *et al.* using fetal rat pituitary primordia, our data showed that low doses of T_3 in combination with corticosterone could further increase somatotroph proportions above the effect of corticosterone alone. On the other hand, we found that high doses of T_3 inhibited the stimulatory effect of corticosterone on somatotroph differentiation. No similar results were reported in other models.

Compared with T_3 , our data demonstrate that all doses of T_4 can act synergistically with corticosterone to augment corticosterone-induced GH cell differentiation *in vitro*. The primary hormone secreted by the thyroid gland is T_4 . Most circulating T_3 is produced by monodeiodination of T_4 in liver, kidney, brain, pituitary, and other peripheral tissues. Type I or II deiodinase activity has been demonstrated in several tissues of fetal rats (38, 39) and embryonic chickens (40), including the pituitary. In cultured GH_3 cells, addition of ipodate, a compound closely related to IOP, caused inhibition of T_4 5'-deiodination and prevented stimulation of GH synthesis by T_4 (41). Our data showed that IOP did not affect somatotroph differentiation induced by corticosterone alone or by the corticosterone/ T_3 combination. In contrast, IOP blocked the slightly stimulatory effect of T_4 and the combined effect of corticosterone/ T_4 . Addition of T_3 reversed the inhibition effect of IOP. Thus, conversion of T_4 to T_3 was necessary for the effect of T_4 on corticosterone induction of somatotrophs, suggesting that T_3 plays the primary role in thyroid hormone regulation of somatotroph differentiation during development. The results of our IOP experiments may also explain the different dose-dependent effects between T_4 and T_3 . Our data indicate that low levels of T_3 acted synergistically with corticosterone to augment corticosterone-induced GH cell abundance. In contrast, the high dose of T_3 (10^{-8} M), which is higher than its physiological level at that age, inhibited the stimulatory effect of corticosterone on somatotroph differentiation. These findings suggest that the capacity of the anterior pituitary to convert T_4 to T_3 is likely limiting at this age of chicken embryonic development, thereby restricting the effective local concentration of T_3 to low levels, even in the presence of high T_4 . If pituitary T_4 monodeiodinase activity is limiting, this would allow for stimulatory effects of thyroid hormones on somatotrophs and limit potentially inhibitory effects of higher circulating T_4 concentrations.

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