Somatotroph recruitment by glucocorticoids involves induction of growth hormone gene expression and secretagogue responsiveness

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Abstract

Prior research indicates that growth hormone (GH) cell differentiation can be induced prematurely by treatment with glucocorticoids *in vitro* and *in vivo*. However, the nature of these responses has not been fully characterized. In this study, the time course of corticosterone induction of GH-secreting cells in cultures of chicken embryonic pituitary cells, responsiveness of differentiated somatotrophs to GH secretagogues, localization of somatotroph precursor cells within the pituitary gland, and the effect of corticosterone on GH gene expression were determined to better define the involvement of glucocorticoids in somatotroph recruitment during development. Anterior pituitary cells from embryonic day 12 chicken embryos were cultured in 10^{-9} M corticosterone for 4 to 48 h and were then subjected to reverse haemolytic plaque assays (RHPAs) for GH. Corticosterone treatment for as short as 16 h increased the percentage of GH cells compared with the control. When corticosterone was removed after 48 h and cells were cultured for an additional 3 days in medium alone, the percentage of GH secretors decreased but remained greater than the proportion of somatotrophs among cells that were never treated with corticosterone. To determine if prematurely differentiated somatotrophs were responsive to GH secretagogues, cells were exposed to corticosterone for 48 h and then subjected to GH RHPAs in the presence or absence of GH-releasing hormone (GHRH) or thyrotropin-releasing hormone (TRH). Approximately half of the somatotrophs induced

to differentiate with corticosterone subsequently released more GH in response to GHRH and TRH than in their absence. The somatotroph precursor cells were localized within the anterior pituitary by culturing cells from the caudal lobe and cephalic lobe of the anterior pituitary separately. Corticosterone induction of GH cells was substantially greater in cultures derived from the caudal lobe of the anterior pituitary, where somatotroph differentiation normally occurs. GH gene expression was evaluated by ribonuclease protection assay and by *in situ* hybridization. Corticosterone increased GH mRNA in cultured cells by greater than fourfold. Moreover, corticosterone-induced somatotroph differentiation involved GH gene expression in cells not expressing GH mRNA previously, and the extent of somatotroph differentiation was augmented by treatment with GHRH in combination with corticosterone. We conclude that corticosterone increases the number of GH-secreting cells within 16 h, increases GH gene expression in cells formerly not expressing this gene, confers somatotroph sensitivity to GHRH and TRH, and induces GH production in a precursor population found primarily in the caudal lobe of the anterior pituitary, a site consistent with GH localization in adults. These findings support the hypothesis that glucocorticoids function to induce the final stages in the differentiation of fully functional somatotrophs from cells previously committed to this lineage.

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Introduction

Differentiation of somatotrophs during embryonic development requires both intracellular and extracellular signals. The pituitary-specific transcription factor Pit-1 is thought to be responsible for the specification of growth hormone (GH) progenitor cells in the fetal pituitary gland (Ingraham *et al.* 1988, Li *et al.* 1990, Castrillo *et al.* 1991, Lin *et al.* 1992). In the rat, GH expression is first detected in the fetal pituitary gland on day 15 of gestation

(Nemeskeri *et al.* 1988, Rodriguez-Garcia *et al.* 1995) but remains at an extremely low level until day 19, when pituitary messenger RNA (mRNA), GH content and the number of somatotrophs increase sharply (Rieutort 1974, Frawley *et al.* 1985, Nogami *et al.* 1989, Rodriguez-Garcia *et al.* 1995). This steep increase in GH expression has been proposed to be induced by the elevation of endogenous glucocorticoid levels. Administration of dexamethasone to pregnant rats induces early GH expression in day 17 or day 18 fetuses (Nogami & Tachibana 1993, Nogami *et al.*

1995), and glucocorticoids have been shown to effectively stimulate somatotroph differentiation in pituitary cultures derived from rats (Hemming *et al.* 1984, 1988, Nogami & Tachibana 1993) and chickens (Morpurgo *et al.* 1997, Dean & Porter 1999).

Our laboratory uses chick embryos as a model to study the regulation of somatotroph differentiation. Somatotrophs first appear in the developing chick by embryonic day (e) 14 and become a significant population by e16 (Jozsa *et al.* 1979, Barabanov 1991, Porter *et al.* 1995a). This ontogenic profile correlates with the capacity of serum from chick embryos to induce GH cell differentiation *in vitro* (Porter *et al.* 1995b), wherein serum from e16 but not e12 can induce the appearance of GH-secreting cells in culture. The GH cell-differentiating activity of e16 chicken serum was attributed to endogenous levels of the adrenal glucocorticoid corticosterone (Morpurgo *et al.* 1997). Concomitant treatment with corticosterone and GH-releasing hormone (GHRH) was shown to amplify the somatotroph-differentiating activity of corticosterone *in vitro* (Dean & Porter 1999), although this effect required more than 3 days of exposure to the hormones. A single *in ovo* injection of e16 chicken serum or corticosterone on e11 was also shown to induce somatotroph differentiation in the chick embryonic pituitary *in vivo* (Dean *et al.* 1999), and this *in vivo* response was restricted to embryos administered corticosterone on e11 or e12 and involved increased expression of GH mRNA in the caudal anterior pituitary (Bossis & Porter 2000), the location of somatotrophs in adult animals.

Although much has been learned regarding the ability of glucocorticoids to induce GH cells from their precursor population, many questions remain, including: (1) what is the time course for glucocorticoid-induced somatotroph differentiation? (2) Is the presence of glucocorticoid necessary to maintain the somatotroph phenotype? (3) Are glucocorticoid-induced somatotrophs responsive to stimulatory hypothalamic secretagogues? (4) Do corticosterone-induced somatotrophs arise in the caudal anterior pituitary, where GH cells normally reside? (5) Does corticosterone-induced somatotroph differentiation *in vitro* involve increased GH gene expression, and if so, (6) is GH mRNA expressed in cells that formerly did not express this gene? The present series of experiments was designed to address these questions using a serum-free culture system and a combination of reverse haemolytic plaque assays for secreted GH, immunocytochemistry for intracellular GH, ribonuclease protection assays for total GH mRNA and *in situ* hybridization for GH mRNA expressing cells.

Materials and Methods

Animals and cell culture

Unless stated otherwise, all cell culture reagents were obtained from Life Technologies (Gaithersburg, MD, USA), and hormones and other chemicals were purchased

from Sigma Chemical Co. (St Louis, MO, USA). All media were supplemented with 0.1% BSA, 100 U/ml penicillin G and 100 µg/ml streptomycin sulphate. Animals used in this study were Single Comb White Leghorn chicken embryos. All procedures with chicken embryos were approved by the Institutional Animal Care and Use Committee. Fertile eggs were placed in a humidified incubator at 37.5 °C. The normal duration of embryonic development for chickens is 21 days. Embryos were removed on e12, and their anterior pituitary glands were isolated with the aid of a dissecting microscope. The pituitaries were dissociated into individual cells by trypsin digestion and mechanical agitation as described previously (Porter *et al.* 1995a). e12 pituitary cells were cultured in serum-free medium consisting of a 1:1 mixture of phenol red-free M199 and Ham's F-12 nutrient mixture supplemented with 0.1% BSA, 5 µg/ml human transferrin, 5 µg/ml bovine insulin, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulphate. Cells were plated in either 12-well tissue culture plates or 60-mm culture dishes coated with poly-L-lysine and allowed to attach for 45 min. Serum-free medium containing the appropriate treatments was then added to a final volume of 2 ml. To determine the time course of corticosterone-induced somatotroph differentiation, anterior pituitary cells (2×10^5 cells/well) were cultured in 12-well plates for 4, 8, 16, 24, 36 and 48 h (37.5 °C; 95% air–5% CO₂) in medium alone or medium containing 10^{-9} M corticosterone. This concentration of corticosterone was chosen because it was shown to be maximally effective at inducing somatotrophs in our previous work (Morpurgo *et al.* 1997). After each incubation interval, proportions of cells that released GH were determined by reverse haemolytic plaque assay (RHPA) as described below. Cells cultured similarly for 48 h were evaluated subsequently for their ability to release GH in response to 10^{-8} M synthetic human GHRH_{1–40} (hGHRH_{1–40}; Sigma, G8770) and 10^{-8} M thyrotropin-releasing hormone (TRH) for 2, 4, 8, and 20 h by RHPA. To determine whether continuous exposure to corticosterone was necessary to maintain increased proportions of GH-secreting cells during extended culture, pituitary cells (5×10^5 /well) were first cultured for 48 h in either medium alone or medium containing 10^{-9} M corticosterone. Then, medium was aspirated from the wells, and the cells were recovered and rinsed thoroughly with Spinner's minimum essential medium (SMEM) and Dulbecco's modified Eagle's medium (DMEM). Subsamples of cells from each treatment were subjected to RHPAs to confirm that corticosterone had induced GH cell differentiation. The remaining cells were cultured for an additional 3 days in either serum-free culture medium alone, medium containing corticosterone (10^{-9} M) or hGHRH_{1–40} (10^{-7} M) alone, or medium containing both corticosterone and hGHRH_{1–40}. These cells were then recovered and subjected to GH RHPAs. To determine whether corticosterone-induced somatotrophs originated

in the caudal lobe of the chick anterior pituitary, the microanatomical location of somatotrophs in adult chickens (Lopez *et al.* 1995), the caudal half and cephalic half of e12 pituitary glands were dissociated separately along with entire anterior pituitary glands from other embryos, and the resulting cells from the caudal lobe, the cephalic lobe and the entire anterior pituitary gland were treated for 24 h with 10^{-9} M corticosterone. GH-containing cells were identified by immunocytochemistry as described below. To determine the effect of corticosterone on GH mRNA levels, anterior pituitary cells ($3\text{--}5 \times 10^6$ /treatment) were cultured in 60-mm dishes containing medium alone or medium plus 10^{-7} M corticosterone for 48 h. Subsets of these cells were then subjected to GH RHPA to confirm that corticosterone had induced somatotroph differentiation. Total RNA was isolated from the remaining cells by the acid-guanidine-phenol-chloroform extraction method (Chomczynski & Sacchi 1987) and analysed by ribonuclease protection assay as described below. To further investigate whether somatotroph differentiation involves induction of GH gene expression, anterior pituitary cells were cultured in medium alone or medium containing 10^{-9} M corticosterone, 10^{-7} M hGHRH₁₋₄₀, or corticosterone in combination with hGHRH₁₋₄₀ for 3 days. After culture, the cells were harvested and subjected to *in situ* hybridization as described below.

Reverse haemolytic plaque assay (RHPA)

The RHPA procedure allows for detection of hormone secretion from individual cells (Neill & Frawley 1983). The assays were performed according to the protocol described in detail previously (Smith *et al.* 1986), using rabbit antiserum against chicken GH and modifications described earlier (Porter *et al.* 1995a). Briefly, recovered anterior pituitary cells (1×10^5 /ml) were mixed with an equal volume of an 18% suspension of protein A-coated ovine erythrocytes and infused by capillary action into previously constructed Cunningham chambers. After cells were allowed to attach for 45 min (37.5 °C, 95% air-5% CO₂), chambers were rinsed with DMEM to remove unattached cells. Then, DMEM containing GH antiserum (1:40) and hGHRH₁₋₄₀ (10^{-7} M) was added to the resulting monolayers of cells. Replicate chambers were incubated for 2, 4, 8 or 20 h as described in the figure legends (3 chambers per treatment per time point). 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 analysed using a light microscope to determine the proportion of GH-secreting cells (plaque formers).

Immunocytochemistry (ICC)

Cultured cells were rinsed once with PBS and fixed in the culture plates with 3.7% formaldehyde in PBS for 10 min.

The cells were then rinsed with PBS, quenched for 10 min with 2% H₂O₂, blocked for 30 min with 1% normal goat serum (NGS), and incubated overnight with rabbit anti-chicken GH (1:8000 in PBS). The cells were then processed using rabbit ABC kits according to the directions supplied by the manufacturer (Vector Laboratories, Burlingame, CA, USA). VIP reagent (Vector Laboratories) was used as substrate for the peroxidase. GH-containing cells were then visualized on an inverted light microscope, and the results expressed as a percentage of all pituitary cells present.

Ribonuclease protection assay (RPA)

After spectrophotometric determination of RNA recovery from dissociated cells, the quality and quantity of the RNA preparations were verified by subjecting each sample to formaldehyde-agarose gel electrophoresis. Ethidium bromide staining was used to assess RNA degradation and verify content in each sample. Samples used were those for which no evidence of RNA degradation was apparent. Total cellular RNA was then subjected to the GH RPA. The construct used for riboprobe synthesis was produced in this laboratory. A chicken GH (cGH) cDNA (Lamb *et al.* 1988) was provided by Dr Doug Foster, University of Minnesota. This plasmid was digested with EcoRI and XbaI, and the resulting cGH cDNA fragment was ligated into the EcoRI and XbaI sites of the pGEM-4Z plasmid vector (Promega, Madison, WI, USA). The resulting plasmid (cGH-AS) was digested with EcoRI, and a 320-base ³²P-labelled cGH antisense riboprobe synthesized with T7 RNA polymerase and the MAXIscript labelling kit (Ambion, Austin, TX, USA). The RPA was performed using the RPA II kit (Ambion) according to the procedure provided by the supplier. Total cellular RNA (3 to 15 µg) and ³²P-labelled cGH riboprobe (100 000 c.p.m.) were heated (3–4 min at 95 °C) and allowed to hybridize overnight at 45 °C. The samples were then treated for 30 min at 37 °C with 200 µl ribonuclease (RNase) digestion buffer containing 10 U RNase A and 200 U RNase T1. The protected RNA and probe were collected by ethanol precipitation, denatured (95 °C for 3–4 min), and separated by electrophoresis through 8 M urea-5% polyacrylamide gels. Protected bands within the gel were visualized by autoradiography, and relative levels of GH mRNA were determined by video densitometry coupled with image analysis software (ITTI, St Petersburg, FL, USA). The relative levels of GH mRNA were adjusted for micrograms of RNA analysed in 4 separate trials of this experiment.

In situ hybridization (ISH)

Cells cultured as described above were attached to poly-L-lysine-coated slides (6 slides/treatment; 2×10^4 cells/slide), fixed in 4% (wt/vol) paraformaldehyde in 0.01 M PBS for 20 min at room temperature and overnight at

4 °C, UV cross-linked, and washed 3 times in Tris-buffered saline and then once in 2 × saline sodium citrate (2 × SSC). The slides were then dehydrated sequentially in 50%, 70%, 90%, and 100% ethanol containing 0.3 M ammonium acetate. ³⁵S-labelled antisense cGH riboprobe was synthesized as described above for the RPA, substituting ³⁵S-CTP for ³²P-CTP. A negative control sense probe, complementary to the antisense probe, was synthesized with SP6 polymerase and BamHI-digested plasmid. The cells on each slide were hybridized with 1 × 10⁶ c.p.m. probe in 100 µl hybridization buffer (50% formamide, 4 × SSPE (salt sodium phosphate EDTA), 5 × Denhardt's solution, 50 µg/ml yeast tRNA, 50 mM dithiothreitol (DTT) and 5 mM vanadyl ribonucleoside complex) overnight at 45 °C. After incubation, slides were washed in 2 × SSC at room temperature and then treated with RNase A (20 µg/ml) for 15 min at room temperature. Slides were then rinsed five times in 1 × SSC-2 mM DTT and twice in 0.2 × SSC-2 mM DTT at 54 °C. Finally, slides were rinsed in water and dehydrated with 50%, 70%, 90%, and 100% ethanol. Once dry, slides were dipped in photographic emulsion (Kodak NTB2), exposed for 5 weeks at 4 °C, and then developed.

Statistical analysis

The data reported are the least squares means ± s.e. from at least three completely separate experiments with the number of replicates provided in the legend to each figure or in the Results section. For the RHPA, ICC and ISH data, a single percentage of GH-positive cells was determined for each combination of treatments from the replicates for that combination (for a total of at least 300 pituitary cells analysed for each combination in each replicate trial). All data were analysed using the General Linear Models procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Duncan's multiple range test was used for comparisons among treatments. Differences were considered significant at $P < 0.05$.

Results

Time course of somatotroph induction

Exposure to corticosterone for as few as 16 h significantly ($P < 0.01$; $n = 3$) increased the percentage of GH-secreting cells in cultures of e12 pituitaries from $1.8 \pm 0.3\%$ to $4.8 \pm 0.3\%$ of all cells (Fig. 1). Proportions of somatotrophs were also increased following exposure to corticosterone for 24, 36, and 48 h. At 48 h, the percentage of GH-secreting cells following exposure to corticosterone ($8.8 \pm 0.5\%$) was further increased ($P < 0.01$) relative to levels at 16, 24 and 36 h.

Effect of corticosterone withdrawal on proportions of somatotrophs

To determine if continued exposure to corticosterone was necessary to maintain somatotrophs, cells were treated

Figure 1 Time course for corticosterone induction of GH-secreting cells. E12 pituitary cells were exposed to medium alone or medium containing corticosterone (Cort.; 1×10^{-9} M) for 4 to 48 h in culture. After these culture periods, the cells were recovered and subjected to RHPAs for GH in which the cells were incubated with antiserum (1:40) and GHRH (1×10^{-7} M) for 8 h. Results are expressed as percentages of total pituitary cells that secreted GH in the RHPA. The data are the least squares means ± s.e. from three independent experiments. Values denoted with an asterisk are significantly different ($P < 0.01$) from the control value for that time point. The value denoted with a dagger at 48 h is significantly increased ($P < 0.01$) relative to all other values. Identical results were obtained with plaque assays performed for 20 h (data not shown).

with corticosterone for 48 h followed by an additional 72 h with corticosterone, GHRH or no hormonal supplement. Treatment with corticosterone for 48 h increased the proportion of GH secretors to $4.2 \pm 0.4\%$ of all pituitary cells compared with $0.5 \pm 0.1\%$ of cells cultured in medium alone (Fig. 2; $P < 0.05$; $n = 4$). When corticosterone was removed, the percentage of somatotrophs dropped significantly to $2.5 \pm 0.4\%$ of all cells. However, this was still a significantly greater proportion than detected among cells that were never exposed to corticosterone during this experiment ($0.9 \pm 0.2\%$). GHRH was not more effective than medium alone for maintaining the proportion of somatotrophs during the final 3 days of culture. Continuous exposure to corticosterone further increased the proportion of somatotrophs during the final 3 days of culture to $5.4 \pm 0.7\%$ of all pituitary cells. However, the addition of GHRH did not augment the effect of corticosterone on the proportion of somatotrophs during the final 3 days of incubation.

Responsiveness of corticosterone-induced somatotrophs to GHRH and TRH

We next assessed whether somatotrophs induced prematurely on e12 by corticosterone treatment were subsequently responsive to GHRH and TRH stimulation. For

Figure 2 Effect of corticosterone withdrawal on maintenance of induced somatotrophs. E12 pituitary cells were exposed to medium alone or medium containing corticosterone (Cort; 10^{-9} M) for 48 h in culture. Cells were then recovered, rinsed twice in medium, and cultured for an additional 3 days in medium alone or medium containing corticosterone (10^{-9} M), GHRH (10^{-7} M), or corticosterone in combination with GHRH. Cells were then harvested again and subjected to GH RHPA for 8 and 20 h. Results shown are for the 8-h RHPA and are expressed as percentages of total pituitary cells that secreted GH. The presence or absence of corticosterone during the initial 48 h of culture is denoted by the black bars and white bars respectively. The data are the means $\pm$ S.E. from four independent experiments. Treatments with no letters in common are significantly different from one another ($P < 0.05$). Similar results were obtained with plaque assays performed for 20 h (data not shown).

this purpose, plaque assay chambers incubated for 2 and 4 h were evaluated, as these incubation intervals were shorter than that required for maximal plaque formation to occur under basal conditions. Cells were first treated with corticosterone for 48 h and then subjected to the RHPAs with and without secretagogues. The results from this analysis are summarized in Fig. 3. Note that $1.5 \pm 0.5\%$ of all pituitary cells formed GH plaques during the 2-h interval under basal conditions and that GHRH increased this proportion to $4.2 \pm 0.5\%$ of all cells ($P < 0.01$; $n = 3$). During the 4-h interval, $2.1 \pm 0.5\%$ of all cells formed GH plaques under basal conditions, and GHRH increased this proportion to $6.2 \pm 0.5\%$ of all cells ($P < 0.01$; $n = 3$). Given that GHRH increased the percentage of plaque-forming cells by 4.1%, we can conclude that at least this proportion of all pituitary cells or about half of all corticosterone-induced somatotrophs responded to GHRH. Similarly, $4.2 \pm 0.3\%$ of corticosterone-treated cells exposed to TRH for 4 h released GH compared with $1.9 \pm 0.3\%$ of cells exposed to medium alone in the RHPA ($P < 0.05$; $n = 3$). Comparing this increment in the percentage of plaque-forming cells to the maximal proportion of GH-releasing cells detected in this experiment (5.0% of all cells), we can conclude that about half of the somatotrophs present were responsive to TRH.

Figure 3 Responsiveness of differentiated GH cells to GHRH and TRH. E12 pituitary cells were exposed to medium alone or medium containing corticosterone for 48 h in culture. The cells were then harvested and subjected to RHPAs in the absence or presence of GHRH or TRH. Results are expressed as percentages of total pituitary cells that released GH following exposure to 10^{-8} M GHRH (top panel) or 10^{-8} M TRH (bottom panel) for 2 to 20 h in the RHPA. The data are the least squares means $\pm$ S.E. from four experiments for GHRH and three experiments for TRH. Differences between treatment groups within the 2- and 4-h time points, prior to maximal plaque formation under basal conditions, are indicated by asterisks and are significant at $P < 0.05$.

Localization of corticosterone-responsive somatotroph precursor cells within the pituitary

To determine whether corticosterone initiated GH production by cells distributed evenly throughout the anterior pituitary or whether this response was preferentially restricted to one region, cultures of pituitary cells isolated from the cephalic and caudal halves of the gland were treated for 48 h with corticosterone (10^{-9} M). ICC revealed that corticosterone increased the abundance of GH-containing cells in cultures from the caudal lobe from

Figure 4 Corticosterone induction of GH-containing cells in cultures derived from the caudal and cephalic lobes of the anterior pituitary. Anterior pituitaries from e12 embryos were cut into their cephalic and caudal halves, with the resulting cephalic and caudal lobes dissociated separately. The resulting cells, along with cells dissociated from entire pituitary glands of other embryos, were treated for 48 h with corticosterone (Cort; 10^{-9} M). GH-containing cells were identified by ICC. The results are presented as the percentage of all cells present that stained positively for GH and are the least squares means and standard errors from 3 independent trials. Differences between corticosterone and basal treatments are significant at $P < 0.01$ and are indicated by asterisks.

the basal proportion of $19.0 \pm 2.1\%$ to $42.2 \pm 2.1\%$ of all cells (Fig. 4; $P < 0.05$; $n = 3$). In contrast, the abundance of GH cells in cultures from the cephalic lobe following corticosterone treatment ($15.0 \pm 2.1\%$) was not greater than that found for cultures derived from entire intact glands ($19.8 \pm 2.1\%$). The GH cells induced by corticosterone in the cephalic lobe cell cultures may indicate the presence of somatotroph precursors in both lobes on e12. Alternatively, they may reflect a level of contamination of caudal lobe cells in our cephalic lobe cultures. Given the difficulty inherent in cutting an e12 anterior pituitary in half, we cannot rule out the latter possibility. Nonetheless, the abundance of somatotrophs in corticosterone-treated cultures from the caudal lobe was twice that of similar cultures from the cephalic lobe. These results indicate that glucocorticoid induction of somatotrophs occurs primarily in the caudal lobe of the chicken embryonic anterior pituitary gland, where GH-producing cells normally reside in older embryos and in adults.

Involvement of GH gene expression in somatotroph differentiation

RPA and *in situ* hybridization were used to evaluate GH gene expression in pituitary cell cultures. Total RNA was collected from cells treated with corticosterone for 48 h and subjected to RPAs to determine total GH mRNA

Figure 5 Effect of corticosterone on GH mRNA levels and GH cell percentages. E12 pituitary cells ($3-5 \times 10^6$ cells/treatment) were exposed to medium alone or medium containing corticosterone (Cort; 10^{-7} M) for 48 h in culture. Subsets of these cells were then subjected to GH RHPA (see Fig. 1 for further details), while total RNA was isolated from the remaining cells and analysed for GH mRNA levels by RPA. Results shown are the relative levels of GH mRNA as determined by RPA (A and B) and the percentages of total pituitary cells that secreted GH (C). Panel A is an autoradiograph showing results of an RPA in which samples from 2 of 4 separate experiments were analysed. Panel B is the densitometric analysis of the autoradiographs from the 4 trials. The data presented in panels B and C are the least squares means $\pm$ s.e. from the 4 separate experiments. The asterisks indicate that treatment with corticosterone significantly increased ($P < 0.05$) the percentage of GH-secreting cells and the level of GH mRNA.

levels in the cultures. Autoradiographic results from 2 of 4 trials are presented in Fig. 5A, and cumulative results from densitometric analysis of 4 independent trials are presented in Fig. 5B. Corticosterone increased the quantity of

Figure 6 Somatotroph differentiation involves induction of GH gene expression. E12 pituitary cells were exposed to medium alone or medium containing corticosterone (Cort; 10^{-9} M), GHRH (10^{-7} M), or corticosterone in combination with GHRH for 3 days. After culture, the cells were harvested and subjected to *in situ* hybridization with 35 S-labelled, antisense riboprobe to chicken GH. The results are the least squares means $\pm$ S.E. from three separate experiments. Significant differences ($P < 0.01$) among treatment groups are indicated when bars have no letters in common.

protected GH mRNA from the control level of 7.3 ± 5.7 arbitrary densitometric units (ADU) to 26.8 ± 5.7 ADU per μ g total RNA ($P < 0.05$; $n = 4$). In these same trials, corticosterone increased the proportion of GH secretors to $14.2 \pm 2.2\%$ of all pituitary cells compared with $2.4 \pm 2.2\%$ of cells cultured in medium alone (Fig. 5C; $P < 0.01$; $n = 4$). To further investigate whether somatotroph differentiation involves induction of GH gene expression in cells formerly not expressing this gene, e12 pituitary cells were cultured in medium alone or medium containing 10^{-9} M corticosterone, 10^{-7} M GHRH, or corticosterone in combination with GHRH for 3 days. The cells were then subjected to *in situ* hybridization using a 35 S-labelled cGH antisense riboprobe. Proportions of pituitary cells expressing GH mRNA increased from basal levels of $1.6 \pm 0.5\%$ to $5.1 \pm 0.5\%$, $9.7 \pm 0.5\%$, and $13.7 \pm 0.5\%$ for cells cultured with GHRH alone, corticosterone alone, and corticosterone in combination with GHRH respectively (Fig. 6). All treatments were significantly different from one another ($P < 0.01$; $n = 3$ separate experiments). Slides hybridized with the sense probe showed no positive cells. Representative photomicrographs from this series of experiments are presented in Fig. 7.

Discussion

The present series of experiments expands on previous findings and confirms unequivocally that glucocorticoids

can induce the differentiation of functional somatotrophs *in vitro*. Using multiple approaches that assess GH production at several cellular and molecular levels, we demonstrated that corticosterone increased the level of GH gene expression in cultured cells, and the number of cells that expressed GH mRNA, contained GH protein and released GH into the medium. GH secretion from corticosterone-induced somatotrophs was responsive to GHRH and TRH. These findings indicate that corticosterone initiated GH gene expression, augmented GH protein synthesis and induced regulated GH secretion, the hallmark of a functional somatotroph. In all previous studies with either rat or chicken models, glucocorticoid induction of GH cells was determined either following administration *in vivo* to the entire animal (Nogami & Tachibana 1993, Nogami *et al.* 1995, Dean *et al.* 1999, Bossis & Porter 2000) or by using a single approach to define somatotroph differentiation (Hemming *et al.* 1984, 1988, Morpurgo *et al.* 1997, Nogami *et al.* 1997, Dean & Porter 1999). Where glucocorticoids were administered *in vivo*, indirect effects from the maternal endocrine system or other tissues in the developing animal could not be excluded, including the potential for stimulation by hypothalamic GHRH. In approaches using a single technique, the possibility remained that only one aspect of GH production was induced. The current study investigated direct effects of corticosterone on pituitary cells and demonstrated that this glucocorticoid stimulated expression of GH mRNA and GH protein and initiated secretion of GH by a new population of somatotrophs. Considering the body of evidence accumulated to date in rats and chickens, the role of glucocorticoids as extrapituitary factors regulating the final steps in somatotroph differentiation should not be discounted in any model describing GH cell ontogeny.

Corticosterone increased GH mRNA levels in the cultured pituitary cells as detected by RPA, and the increase in GH mRNA in response to corticosterone was due, at least in part, to an increase in the number of cells expressing GH mRNA. This increase in GH mRNA-positive cells and GH mRNA levels could reflect activation of GH transcription and/or stabilization of GH mRNA. The present studies were not designed to distinguish between these possibilities. Glucocorticoids increase pituitary GH mRNA levels *in vitro* in rats (Nogami *et al.* 1997) and humans (Paek & Axel 1987), and these effects are likely mediated, in part, by glucocorticoid response elements (GREs) located in GH genes. The chicken GH gene has been cloned, and its nucleotide sequence including 509 bp of the 5'-flanking region has been determined (Tanaka *et al.* 1992). A potential GRE is present at -215 to -201 of the chicken GH promoter, and potential GREs were also identified in regions of the human (-245 to -206) and rat (-111 to -97) GH promoters (Robins *et al.* 1982, Slater *et al.* 1985, Treacy *et al.* 1991). However, deletion analysis of genes from rats and humans

Figure 7 Representative results from *in situ* hybridization analysis. Cells were treated and analysed as described in the legend to Fig. 6. Shown are the results from a single trial in which e12 pituitary cells were cultured in the presence or absence of corticosterone and GHRH and probed with sense or antisense GH riboprobes. No positive cells were observed with the sense riboprobe. Note that the abundance of somatotrophs detected with the GH antisense riboprobe was greater in cultures treated with corticosterone alone (Cort.) and in combination with GHRH (Cort.+GHRH).

indicated that sequences mediating glucocorticoid regulation of GH gene expression are primarily located downstream from the transcriptional initiation site (Robins *et al.* 1982, Slater *et al.* 1985, Birnbaum & Baxter 1986, Treacy *et al.* 1991). A potential GRE is also present within the first intron of the human GH gene (Slater *et al.* 1985) and in a similar position in the first intron of the chicken GH gene. The present findings indicate a fairly rapid induction of somatotroph differentiation that is delayed by less than 16 h. This amount of time would be sufficient to induce GH gene expression, protein synthesis, hormone packaging and release. Induction of each of these events is indicated by our present results. The length of time required for corticosterone to augment GH cell abundance

would also be sufficient for the production of intermediate factors that could, in turn, induce GH cell differentiation. Protein synthesis is required for premature induction of GH mRNA in cultured fetal rat pituitary cells (Nogami *et al.* 1997), supporting the involvement of an intermediary protein. Unpublished results in our laboratory have confirmed this in cultured pituitary cells from embryonic chickens. Thus, the induction of GH mRNA by glucocorticoids that is associated with somatotroph differentiation might not be a direct effect of glucocorticoids on the GH gene, but an effect mediated by a factor produced in the pituitary in response to glucocorticoids. The nature of this protein and whether it is produced within the presomatotrophs or another cell type is not known.

Pituitary GH release in chickens, as in other vertebrates, is controlled by stimulatory and inhibitory factors from the hypothalamus. GHRH and TRH are thought to be the primary stimulatory GH secretagogues in chickens. In previous studies, 50 to 70% of initial somatotrophs present on e16 released GH in response to GHRH (Porter *et al.* 1995a, Dean *et al.* 1997), while only 30% of e16 GH cells were responsive to TRH (Dean *et al.* 1997). By e20, proportions of somatotrophs that responded to GHRH and TRH were approximately equal. Following treatment of e12 pituitary cells with corticosterone in the present study, at least 50% of somatotrophs present were responsive to GHRH and TRH. Thus, somatotrophs induced prematurely by treatment with corticosterone on e12 are at least as responsive to GHRH and TRH as GH cells that differentiate normally by e16. In addition, corticosterone induction of somatotrophs occurred primarily in cultures from the caudal lobe of the anterior pituitary. While many GH-containing cells were induced in cultures derived from the cephalic lobe, nearly half of all cells obtained from the caudal lobe produced GH in response to corticosterone. The caudal lobe of the anterior pituitary is the same region where somatotrophs are located in adults (Lopez *et al.* 1995) and where GH mRNA is expressed prematurely in response to corticosterone administration *in vivo* (Bossis & Porter 2000). Together, these findings indicate that the corticosterone-induced population of GH cells likely reflects those cells that were destined to differentiate into somatotrophs at a later age and that the induced somatotrophs are fully functional in their responses to GHRH and TRH. It follows that the induced GH cells were already committed to the somatotroph lineage, and that corticosterone functions as the final signal to induce the mature somatotroph phenotype.

Previous work by our group established that, in contrast to corticosterone, treatment of e12 chicken pituitary cells with GHRH for as many as 6 days failed to increase the percentage of GH-secreting cells (Porter *et al.* 1995b, Dean & Porter 1999). This finding was surprising, because GHRH has been shown to stimulate proliferation of rat somatotrophs *in vitro* (Billestrup *et al.* 1986). However, treatment of e12 chicken pituitary cells with corticosterone in combination with GHRH resulted in a greater stimulation in the proportion of somatotrophs than treatment with corticosterone alone (Dean & Porter 1999). In the present study, combined treatment with GHRH and corticosterone resulted in more GH mRNA-positive cells than treatment with corticosterone alone. Treatment of e12 chicken pituitary cells with GHRH alone for 3 days increased the percentage of GH mRNA-positive cells 3.2-fold over basal levels, while treatment with corticosterone alone was nearly twice as effective, increasing the percentage of positive cells to 6.1 times that of controls. Corticosterone and GHRH exerted an additive effect, as treatment with both hormones increased the proportion of GH mRNA-positive cells to 13.7% of all pituitary cells, an

8.6-fold increase over the control level. These results, together with previous findings, indicate that GHRH is effective at increasing GH mRNA expression in existing somatotrophs and may act in conjunction with glucocorticoids to induce GH gene expression during somatotroph differentiation. However, we also found that withdrawal of corticosterone for 3 days diminished the abundance of somatotrophs, although not to proportions as low as those found in cultures never treated with corticosterone. Exposure to GHRH during the corticosterone withdrawal period failed to maintain the increased proportion of somatotrophs induced by corticosterone. Thus, corticosterone-induced increases in the proportion of somatotrophs are at least partially reversible and apparently dependent upon the continuous presence of corticosterone. This finding would indicate that glucocorticoids act as both a differentiating factor and as a trophic factor for somatotrophs during embryonic development that cannot be substituted for by GHRH.

Our present findings support the hypothesis that glucocorticoids induce somatotroph differentiation. Analysis by *in situ* hybridization, immunocytochemistry and reverse haemolytic plaque assays indicated a corticosterone-induced increase in the abundance of GH cells. An alternative interpretation of these findings is that the trophic actions of corticosterone merely increased GH gene expression, protein production and hormone release by a population of existing somatotrophs above the levels of sensitivity for each of these assays. However, it seems unlikely that each of these sensitive techniques was unable to detect this population of somatotrophs. Furthermore, in our previous reports (Porter *et al.* 1995b, Morpurgo *et al.* 1997, Dean & Porter 1999) treatment with GHRH or TRH, the principal stimulatory GH secretagogues in chickens, for 2 to 6 days failed to increase the abundance of GH-secreting cells in cultures of embryonic pituitary cells. Similarly, the aqueous phase of ether-extracted embryonic serum failed to induce GH cell differentiation (Morpurgo *et al.* 1997). In contrast, the ether phase effectively induced GH-secreting cells, and the somatotroph-differentiating activity in the embryonic serum was found to be corticosterone. Currently, we found that somatotroph induction in response to corticosterone is rapid, occurring within 16 h. Thus, glucocorticoids would appear to be more than a mere trophic regulator of GH expression. Rather, they seem to be a requisite factor for final expression of the somatotroph phenotype, a role that cannot be substituted for by either GHRH or TRH. However, our current findings do indicate that somatotrophs induced by corticosterone are responsive to both TRH and GHRH. Either TRH and GHRH receptors were already present on these cells prior to corticosterone induction of GH, or corticosterone induced their expression in addition to GH. Whether glucocorticoids act to change the fate of a progenitor population into the somatotroph lineage or, instead, act as

a final signal regulating the differentiation of functional somatotrophs from cells already committed to this lineage is difficult to address. Our findings that the corticosterone response occurs within 16 h, that these cells arise predominantly in their normal microanatomical location, and that they respond as normal somatotrophs to TRH and GHRH tend to favour the latter possibility. We conclude that glucocorticoids may function as a requisite signal for induction of the final stages of somatotroph differentiation from cells that are already committed to this lineage.

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