ABSTRACT

Title of Thesis: SOMATOTROPIN RESPONSE TO CORTICOSTERONE AND THE THYROID HORMONE T_3 DURING CHICK EMBRYONIC DEVELOPMENT: INVOLVEMENT OF TYPE I AND TYPE II GLUCOCORTICOID RECEPTORS

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Corticosterone (CORT) can stimulate growth hormone (GH) cell (somatotroph) differentiation and GH secretion on embryonic day (e) 12 but not e20 in the chicken. GH induction involves both glucocorticoid receptors (GR) and mineralocorticoid receptors (MR); however, this finding has been characterized only on e12. To further define changes in somatotroph responsiveness to CORT, pituitary cells obtained on e12-e20 were cultured with CORT alone and in combination with T_3. GH secretion increased over basal with CORT treatment on e12, e14, e16, and e18, but not e20. Contributions of GR and MR in CORT responses were evaluated using GR and MR antagonists. Blocking both receptors was required to abolish the CORT response by e12 cells. The same treatment on e20 decreased GH secretion relative to basal. We conclude that positive somatotroph responses to CORT are lost during embryonic development and that both GR and MR mediate CORT-induced GH secretion by cultured somatotrophs.

SOMATOTROPIN RESPONSE TO CORTICOSTERONE AND THE THYROID HORMONE T_3 DURING CHICK EMBRYONIC DEVELOPMENT: INVOLVEMENT OF TYPE I AND TYPE II GLUCOCORTICOID RECEPTORS

By

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LIST OF ABBREVIATIONS

Ab	antibody
ALDO	aldosterone
BSA	bovine serum albumin
CORT	corticosterone
CV	coefficient of variation
d	day
Da	Dalton
DMEM	Dulbecco's modified Eagle's medium
e	embryonic day
ELISA	enzyme linked immunosorbent assay
h	hour
GH	growth hormone
GHRH	growth hormone-releasing hormone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
ICC	immunocytochemistry
IgG	immunoglobulin G
K_d	dissociation constant
M	molar
mAb	monoclonal antibody
mL	milliliter
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
µg/mL	micrograms per milliliter
ng/mL	nanograms per milliliter
NGS	normal goat serum
nm	nanometer
nM	nanomolar
PBS	phosphate buffered saline
RPA	ribonuclease protection assay
RHPA	reverse hemolytic plaque assay
SFM	serum free culture media
SPIRO	spironolactone
TA	triamcinolone acetonide
TMB	tetramethylbenzidine
TR	thyroid hormone receptor
T_4	thyroxine
T_3	triiodothyronine
ZK98	ZK98299

Chapter 1 Literature Review

Introduction

The anterior pituitary gland is composed of five distinct types of cells, all of which produce and secrete a specific hormone. Somatotrophs, corticotrophs, gonadotrophs, thyrotrophs and lactotrophs secrete growth hormone (GH), adrenocorticotropic hormone (ACTH), luteinizing and follicle-stimulating hormones, thyroid-stimulating hormone (TSH) and prolactin, respectively (Griffen & Ojeda, 2000). Corticotrophs are the first pituitary cell to differentiate, followed by gonadotrophs, thyrotrophs, somatotrophs and finally lactotrophs.

Research conducted in our lab focuses on the differentiation of somatotrophs in chickens and the extrapituitary signals that regulate this phenomenon. The glucocorticoid, corticosterone (CORT), is the extrapituitary signal that initiates somatotroph differentiation in chickens. Somatotroph differentiation and the regulation of GH secretion are also regulated by the thyroid hormones. The chicken is an ideal model to study anterior pituitary development because maternal interactions are not a factor as in mammals. The egg also allows for easy manipulation of the embryo for a multitude of experiments.

In order to study somatotroph differentiation in the anterior pituitary gland of the chicken, several methods have been employed extensively. Each one has advantages and disadvantages, but more importantly, each one reveals a slightly

different angle to the overall picture of the differentiation of the anterior pituitary gland.

Whole tissue mount *in situ* hybridization is a method to detect the presence of a mRNA at the tissue level. To detect chicken GH, construction of a riboprobe is necessary, and incubation with the specific riboprobe produces distinct staining in the tissue if chicken GH is present. It is a method useful for making conclusions at the whole tissue level after manipulating the entire embryo. It does not provide information at the single cell level.

Reverse hemolytic plaque assays (RHPA) allows for the detection of protein secretion from a single cell. It is a means of correlating the plaque area produced to the amount of a specific protein, for instance GH, secreted. This assay can only detect the presence of the protein if it has been secreted; it lends no evidence to the amount of protein remaining inside of the cell.

Immunocytochemistry (ICC) is an assay to detect the intracellular stores of a protein, specifically GH in this case. Cells are attached to glass slides, fixed, and then incubated with a GH polyclonal antiserum. Cells containing GH stain dark, while those that do not remain clear. This type of assay clearly demonstrates the population of somatotrophs in a sample. It is not able to determine the amount of GH present or the rate of secretion by somatotrophs. However, when used in combination with an RHPA, both protein secretion and production can be explored.

The previous assays can only detect if the protein is present at the single cell level. None of the assays give an exact estimate of the concentration of protein

secreted. The radioimmunoassay (RIA) is a method that directly estimates the amount of a given protein in a sample. It can be used to quantify a protein in cell culture medium, blood plasma and serum, urine, extracellular fluid or cellular fractions. The protein of interest competes with a radioactively labeled tracer for its cognate receptor. The amount of protein in the sample is inversely proportional to the amount of radioactivity counted. RIA, although highly accurate and able to quantitatively estimate the amount of protein present, requires radioactivity training and is potentially hazardous to the researcher's health. Enzyme-linked immunosorbent assay (ELISA) works similarly, but instead of using radioactivity, it uses the principles of protein binding and enzymatic activity to estimate the amount of protein in a sample. An ELISA can be completed in one day. The research centered on somatotroph differentiation of the embryonic chicken conducted in our lab has exclusively used the methods described previously.

Chicken somatotrophs differentiate around e14. Administration of CORT can induce the premature appearance of somatotrophs in cultures of embryonic chicken pituitary cells as seen with RHPA and ICC (Porter *et al*., 1995a). By late embryonic development, existing somatotrophs are no longer responsive to CORT when administered during the RHPA (Dean *et al*., 2001). Because these two observations were made using two different approaches, it is difficult to conclude that this apparent change in responsiveness has an underlying reason. CORT binds with both the type I glucocorticoid receptor (MR) and the type II glucocorticoid receptor (GR) to affect gene transcription. The apparent change in responsiveness of

somatotrophs to CORT may be due to a change in expression or availability of the two types of receptors.

Growth Hormone

GH is a 191-amino acid protein with a molecular weight of 22,650 Da that is structurally related to human chorionic somatomammotropin, a peptide produced by the placenta (Griffen & Ojeda, 2000). GH has species specificity due to differences in molecular structure, and it is stored in secretory granules in somatotrophs. The GH produced by somatotrophs actually accounts for between 4-8% of the gland by dry weight. One of the transcription factors, Pit-1, that regulates the GH gene has was isolated in the chicken pituitary (Van As *et al*., 2000). It has 84% homology with mouse Pit-1 and 97% homology with rat Pit-1. The transcription factor was detectable in embryonic chicken from e5 onwards.

Growth hormone releasing hormone (GHRH) is a 44-amino acid peptide encoded by a single gene that is primarily localized to the arcuate nucleus and the median eminence of the hypothalamus (Griffen & Ojeda, 2000). Its production in the hypothalamus stimulates the release of GH from the anterior pituitary. Administration of GHRH can stimulate cultured chicken somatotrophs to release their stored GH *in vitro*, as seen with reverse hemolytic plaque assays (RHPA) (Porter *et al*., 1995). The effects of GHRH are readily seen after 4 hours and become maximal at 8 hours. Secretion of GH from embryonic chicken somatotrophs is also regulated by thyrotropin-releasing hormone (TRH), somatostatin, and insulin-like growth factor

1 (IGF-1), as well as ghrelin and GH secretogogue (GHS), although GHRH is still the most potent (Dean *et al*., 1997).

Metabolic signals also control GH secretion (Griffen & Ojeda, 2000). When exogenous insulin is administered to humans to mimic hypoglycemia results in immediate GH upregulation, as does the amino acid arginine when taken orally. Conversely, non-esterified fatty acids reduce GH secretion, although the mechanism is not well understood.

Corticosterone (CORT)

CORT is a steroid hormone secreted by the cortex of the adrenal gland. It is considered a glucocorticoid and aids in regulation of the conversion of amino acids into carbohydrates and glycogen by the liver. It also assists in the stimulation of glycogen formation in other tissues. One of the enzymes responsible for CORT conversion from cholesterol is 3β-HSD (Kanda *et al*., 2000). The mRNA that codes for this protein was expressed in the chicken embryonic adrenal glands beginning on e5. It had continued expression until e12 when the study was terminated. The mRNA for another enzyme, P450scc, was expressed in the adrenal glands beginning on e7.

The SF-1/Ad4BP gene is the major regulator controlling steroidogenic P-450 genes (Kudo & Sutou, 1999). It belongs to the steroid receptor superfamily and is expressed in the adrenal cortex, gonads and pituitary gonadotroph. It plays a critical role in the development of the steroidogenic tissues and pituitary gonadotrophs.

CORT is similar in structure, although somewhat less potent, than the other glucocorticoids, cortisol and cortisone (Griffen & Ojeda, 2000). It is produced in response to stimulation by the pituitary hormone ACTH. In chickens and rats, but not in humans and mice, CORT is the predominant glucocorticoid secreted by the adrenal

gland. It is a precursor in the synthesis of aldosterone, another adrenal cortical steroid.

CORT, and glucocorticoids in general, first stimulate and then inhibit GH secretion in a dual manner (Griffen & Ojeda, 2000). CORT is involved in many processes and is essential for life. The process of gluconeogenesis, where protein in muscle is converted into glucose for energy, requires the presence of CORT. CORT then decreases the utilization of glucose by cells. It is necessary for protein and lipid metabolism, and it is involved in maintaining normal vascular integrity as well as suppressing inflammation. In the central nervous system, CORT modulates perception and emotion.

The Thyroid Hormones: T_3 and T_4

The two principal thyroid hormones are thyroxine (3,5,3',5'-tetraiodo-L-thyronine; T_4) and triiodothyronine (3,5,3'-triiodo-L-thyronine; T_3). The amino acids diiodotyrosine and monoidodotyrosine serve as the precursors for the synthesis of these hormones. The more active form, T_3, is formed by removing an iodine group from thyroxine, catalyzed by 5'-deiodinase. Plasma T_4 can be detected as early as e9 in the chicken with levels around 2 ng/mL (Scanes *et al*., 1987). Plasma T_4 levels rise steadily throughout chicken embryonic development with a sharp increase (>20 ng/mL) prior to hatch. Plasma T_3 levels decrease from >2 ng/mL to <1 ng/mL from e9 to e11 and then remain low (~1 ng/mL) and relatively constant until e19 when they rise dramatically to >5 ng/mL. While the primary regulator of thyroid gland function is pituitary TSH, the hypothalamus also controls thyroid function to a lesser extent through release of thyrotropin releasing hormone (TRH).

Thyrotrophs appear earlier in chicken embryonic development than somatotrophs, on e9, and become evenly distributed by e20 (Sasaki *et al*., 2003). Thyroid hormones are thought to enter target cells by passive diffusion, in a manner similar to steroid hormones (Griffen & Ojeda, 2000). However, there is new evidence that a carrier system is used and that the receptors are anchored to the nucleus through nonhistone nuclear proteins to facilitate gene transcription. The

thyroid hormones function to increase basal metabolism. Studies in mammals demonstrating the combined effects of glucocorticoids and the thyroid hormones have shown that these two agents act synergistically (Samuels *et al*, 1979; Vale *et al*, 1983; Williams *et al*, 1991).

Development of the Chicken Anterior Pituitary Gland

Differentiation of Somatotrophs

In chickens, GH mRNA in the cephalic lobe increases from e16 and reaches maximum on e20 (Kansaku *et al*., 1994). Similar results were seen in the caudal lobe, with more GH mRNA localized to the caudal lobe. After embryonic development, GH levels do not vary from pre-laying, laying, 1-week incubation, 3-week incubation and brooding. Again, during these life stages, caudal lobe GH mRNA was significantly higher than that in the cephalic lobe, although both lobes showed no life-stage effect.

Glucocorticoids from the adrenal gland stimulate the differentiation of somatotrophs in the anterior pituitary gland. Our group has previously shown that somatotrophs become a significant population of chicken anterior pituitary cells between embryonic day 14 and embryonic day 16 (e14-e16) (Porter *et al*., 1995a), although a few appear as early as e12. Somatotrophs in culture do not differentiate autonomously. Serum taken from e16 embryos can induce somatotrophs to differentiate in vitro. However, serum taken from e12 embryos cannot induce somatotroph differentiation in vitro. Further, cell division was not necessary for induction of somatotroph differentiation (Porter *et al*., 1995b). The blood-borne signal was determined to be CORT (Morpurgo *et al*., 1997). The most effective dose of CORT to induce somatotroph differentiation was found to be 2.5 nM.

CORT not only induces the appearance of somatotrophs in cell culture, but also in living embryos. In developing embryos, CORT increased significantly between e14 and e15 from 11.3 ng/mL to 20.4 ng/mL as measured by RIA, and CORT remained at this concentration until e20 when a significant decrease was observed (Kalliecharan & Hall, 1974). Other adrenal steroids, such as cortisol, peaked at e15 in the chicken with 22.3 ng/mL, while cortisone peaked at e17 with 25.7 ng/mL. Nonadrenal steroids, such as progesterone, exhibited a steady increase from e9 to e20 with a peak at e20 with 31.2 ng/mL, a different profile than glucocorticoids.

An alternative study found that serum CORT levels in the embryonic chicken was 3.6 ng/mL on e10 and three times as much (9.0 ng/mL) on e20, determined by RIA (Scott *et al*., 1981). CORT steadily rose during the last half of incubation. In this study, embryos were staged according to their middle toe length, an important indicator of developmental age. Serum levels of CORT on e10 through e16 were statistically different from the previous day. However, serum levels on e16 through e20 were not statistically different from the previous day.

Exogenous treatment of CORT has different effects on the developing chicken embryo (Mashaly *et al*., 1991). The dose of CORT injected into the egg was determined by weighing of the dry egg before and after injection. All CORT treatments (0.1%, 0.05%, 0.01%) decreased embryo body weight, and embryos treated with 0.1% CORT (the highest dose) died by e21. The mortality rate was highest between e19 and e21 for all treatment groups. The mortality rate did not

differ among treatment groups from 4 to 10 days, except for e6, which was higher. The control group and 0.01% CORT treatments had lower mortality than the other treatment groups. There was an observable increase in serum CORT levels on e19. Specifically, a sharp peak in serum CORT levels occurred at e19 in the 0.01% and 0.05% treatment groups, and a sharp peak occurred at e17 in the 0.1% treatment group. The 0.1% CORT treatment group had significantly lower overall serum CORT levels. The dose of CORT was determined by weight, which may have been too high of a dose to accurately mimic physiologically concentrations. Plus, CORT was given on e0, an age where steroids have proven to be lethal. This is evidence that low doses of CORT may work in a positive feedback manner increasing GH levels at appropriate times during development, while higher doses of CORT may work in a negative feedback manner decreasing GH levels later in development.

The Effects of CORT and GHRH on Somatotroph Differentiation

CORT is known to induce chicken somatotroph differentiation *in vitro*, and GHRH induces the cultured GH cells to release their stored GH (Dean *et al*., 1997). GHRH significantly increases the amount of GH-secreting cells at 10 nM and 100 nM on e16 and e18 after an 18 h treatment. E20 cells are responsive to GHRH at doses from 0.01 nM to 100 nM. This may be due to the already high levels of GH stored in the cells. Sensitivity to GHRH increases in an age-dependent manner between e16 and e20.

The combination of CORT and GHRH significantly increased the amount of GH secreted by e12 anterior pituitary chick embryonic cells into the cell culture medium after two and six days, while neither agent alone had any measurable effect (Dean & Porter, 1999). The same treatments as measured by RHPA showed that CORT was able to increase the number of GH-secreting cells alone, and the addition of GHRH had no effect after two days, but significantly increased the number of GH-secreting cells after six days in culture. As stated previously and reconfirmed with these results, CORT induces somatotroph differentiation and increases the number of somatotrophs in culture, while GHRH induces the cells to release their stored GH into the surrounding environment.

Later in development, somatotrophs do not respond as quickly to CORT, but respond similarly to GHRH as seen on e12 cells. Cells obtained from e17 pituitaries were able to respond to GHRH alone, however CORT had no effect after two days in culture as measured by the amount of GH secreted into the cell culture medium (Dean & Porter, 1999). After six days in culture, GHRH alone had no effect on the amount of GH-secreting cells, but the administration of CORT alone and in combination with GHRH significantly increased the amount of GH-secreting cells.

The effects of CORT on somatotrophs are not only seen in chickens, but also in mammals. Glucocorticoids induced somatotroph differentiation in fetal rats *in vitro* using explants of fetal pituitary primordia obtained on day 14 of gestation (Hemming, 1988). In cultured rat pituitary cells obtained from 13.5 day old fetal rat pups and maintained in culture for 8 days, cortisol stimulated the differentiation of GH-cells (Sato & Watanabe, 1998). Similar effects were seen in 16.5 day old fetal cells that were maintained in culture for 5 days.

The effects of CORT in chickens are also seen at the whole embryo level by treating embryos in ovo on e11, a few days before normal somatotroph differentiation (Bossis & Porter, 2000). The somatotroph population as measured by RHPA increased significantly by e13 with a treatment dose of 0.2 μg and 2 μg. The same increase in the number of GH secreting cells was seen when treating embryos on e12 and examining on e14. But the observed increase in the number of somatotrophs did not persist later in development (e16) through post-hatch day (d) 1. The same doses

administered on e8 and e9 and then examined two days later did not result in an increase in the number of somatotrophs. Treatment of 2 μg and 20 μg on e0 resulted in full mortality between e4 and e6. Treatment of 20 μg on e8, e9 and e10 also resulted in some mortality, with a higher incidence of mortality occurring when the embryos were injected at a younger age. Incidentally, treating with lower amounts of CORT on these same ages had no effect. Clearly, there is a predetermined timeline of when somatotrophs are responsive to CORT, between e11 and e12. Administration of CORT too early in development results in embryonic death and when given later in development, the cells are already partially differentiated and not responsive to CORT.

The effects of CORT and GHRH on somatotroph differentiation involves induction of growth hormone gene expression (Porter *et al*., 2001a). Somatotroph abundance was increased after as little as 16 h with CORT treatment. After 48 h, CORT was able to almost double the percentage of somatotrophs over the 16 h treatment group. When CORT was removed from embryonic serum with an antibody, the induction of somatotrophs decreased. However, the amount of somatotrophs was still higher than if the cells had never been exposed to CORT at all. This finding is strengthened with the fact that administration of CORT directly increases GH mRNA. Addition of GHRH did not augment the effect of CORT, but it was able to induce GH secretion after 2 and 4 h, although not after 8 or 20 h. TRH had a similar effect as GHRH.

Although CORT increases GH mRNA, the induction of GH gene expression is delayed and requires protein synthesis (Bossis & Porter, 2003). But the identity of the protein(s) required is not known. Induction of GH gene expression may involve one or several signal transduction cascades. Through the use of signaling cascade enzyme inhibitors, it was found that the PKA and PKC signal transduction cascades do not mediate the effects of CORT. On the other hand, a RAS GTPase is involved in this process. Further, this is independent of MAPK activation (Porter & Bossis, 2003).

It has been established that administration of CORT induces chicken somatotroph differentiation on e12, resulting in an increase in GH secretion. Premature somatotroph differentiation induced by CORT has been well established using RHPA to measure both the numbers of cells containing GH and the relative amount of GH secreted into the extracellular space. ICC and RIA have also been employed to elucidate the ontogeny of GH secreting cells in culture. However, when 1 nM of CORT was administered to e16, e18, and e20 cells in culture, there was no effect on the number of GH-secreting cells after 24 h of incubation (Porter & Dean, 2001). Using RHPA, it was difficult to determine the optimum amount of time in culture so that noticeable effects in GH secretion could be detected. After e16, constitutive GH secretion produces plaques in the RHPA around almost all somatotrophs. By examining GH secretion after 6, 12, and 18 h in culture, the differences in the effects of CORT, if any, could not be determined. No significant differences were seen after 6 h in culture in e16, e18, and e20 cells. After 12 h of

incubation, no effect was seen with e16 and e18 cells, however, this treatment was slightly stimulatory on e20. Alternatively, after 18 h of incubation, CORT did produce an increase of GH with e16 and e18 cultured somatotrophs, but not e20. During the final quarter of embryonic development, basal levels of GH were almost maximal. This could be why CORT was unable to elicit a large response after e16. This apparent change in responsiveness to CORT over the last half of embryonic development warrants a more thorough investigation because the studies using e12 and e16, e18, and e20 were conducted under slightly different culture systems, times, CORT concentrations and assay methods.

The Effects of CORT and the Thyroid Hormones on Somatotroph Differentiation *in vivo* and *in vitro*

The thyroid hormones, T_3 and T_4, are also able to regulate somatotroph differentiation. Pax8-/- mice are born athyroid to euthyroid dams and have a deletion in the paired box transcription factor Pax8 (Friedrichsen *et al*., 2004). These mice are an ideal model to study congenital hypothyroidism. In 21 day old Pax8-/- mice, expression of GH was significantly reduced compared to wild-type littermates. The expression of GH mRNA and protein in the anterior pituitary was greatly reduced. When the mice were treated with 20 ng of T_4 per gram of body weight, GH expression increased to wild-type levels.

In chickens, T_3 significantly reduced GH secretion by e16 cells after 16 hrs in culture. Again, there was no effect of T_3 on e18 or e20 (Porter & Dean, 2001). The combination of CORT and T_3 was inhibitory on e16-e20, with secretion of GH by e16 somatotrophs being most affected. Subsequently, it was found that T_3 has a biphasic, dose-dependent, effect on the abundance of GH-secreting cells in cultures of e11 pituitary cells (Liu *et al*, 2003). At low doses ($10^{-11}$and 10^{-10} M), GH cells increase beyond the observed CORT response. However, at higher doses ($10^{-9}$and 10^{-8} M), the CORT response was suppressed. This was seen after culturing for both 3 days and 6 days. It was also revealed that T_4 must be converted to T_3 for modulation of somatotroph abundance by T_4.

In rats, when the dam is given T_3 and T_4, plasma GH concentrations and pituitary somatotroph numbers of the fetus are increased (De Escobar *et al*., 1993). This effect is only seen late-term at 18 to 21 days of gestation. T_3 has also been shown to increase GH levels in fetal day 19 rats (Rodriguez-Garcia *et al*., 1995).

The Effects of Synthetic Glucocorticoids (Dexamethasone) on Somatotroph Differentiation

Dexamethasone (DEX) is similar to CORT in structure and action. Pregnant rats were given DEX in their drinking water for 40 hrs before sacrifice (Nogami *et al*., 1993). This treatment increased GH cells in the anterior pituitary gland of the fetus on fetal day 18. The observed effect was decreased earlier in development, as seen in the diminished effect observed on day 17 and the nonexistent effect on day 16. Administration of DEX to the pregnant mother in her drinking water elicited an induction of GH-cell differentiation in the fetal rat pups. Normally, somatotroph differentiation is not observed until e18, and under these conditions, somatotrophs differentiated on e17. However, injection of DEX failed to produce these same results.

Administration of DEX and T_4 augmented the previously observed increase in somatotroph differentiation (Nogami *et al*., 1995). The addition of methimazole, an agent that prevents the conversion of T_4 to T_3, abolished these observed effects.

DEX increases GH mRNA-containing cells obtained from e18 rat pituitary cells; however, the increase observed never exceeded that of intact e19 rat pituitary cells (Nogami *et al*., 1997). The same treatment on e16 did not show this, implying that somatotrophs are responsive to glucocorticoids at distinct time points during development. The DEX-induced increase of GH-containing cells was not observed

after 5 or 10 h and was only observed after a full 24 h. As in chickens, the addition of a protein synthesis inhibitor completely abolished the DEX-induced increase in GH positive cells.

Type I (Mineralocorticoid) and Type II (Glucocorticoid) Receptors in Relation to CORT regulation of Somatotroph Differentiation

Mineralocorticoid and Glucocorticoid Receptors

Both GR and MR belong to the steroid/thyroid/retinoid/orphan (STRO) receptor superfamily. This superfamily of receptors has over 150 members, including the progesterone receptors (PR) and androgen receptors (AR). GR and MR bind to hormone response elements (HRE) and regulate transcription (Funder, 1997). Both of these receptors consist of three functional domains: the carboxy-terminal ligand binding domain (LBD), the central DNA-binding domain (DBD), and the amino terminus transactivation domain. The amino terminus varies in size and displays no sequence conservation between the two receptors (Rupprecht *et al,* 1993). The GR and MR differ by only three AA in their DNA binding domains (Berger *et al*., 1996). Valine 571 infers specificity between the MR and GR, preferentially binding glucocorticoids over mineralocorticoids (Lind *et al*., 1999).

The human glucocorticoid receptor (GR) has two forms: a ligand-binding GRα of 777 amino acids and a 742-amino acid β isoform (Funder, 1997). The β isoform differs only in the last fifteen amino acids and does not bind active glucocorticoids. GRβ is expressed at varying levels in a range of tissues and may act as a ligand-independent negative regulator of glucocorticoid action. Some reports

show that GRα exhibits high affinity for dexamethasone, modest affinity for cortisol and corticosterone and low affinity for aldosterone (Funder, 1997). The human mineralocorticoid receptor (MR) consists of 984 amino acids and has 57% amino acid identity with GRα in the ligand binding domain and 94% in the DNA binding domain.

GR and MR are located throughout the body, in both epithelial and non-epithelial tissues, such as the kidney, colon, salivary glands and heart (Griffen & Ojeda, 2000; Funder, 1997). Substantial amounts of both GR and MR are localized in the anterior pituitary. MR also has significant levels in the hippocampus and the septum, while GR has high levels in the cerebellum. Only small amounts of GR are found in the renal medulla (Reul *et al*, 1990).

The main difference reported between these two receptors, besides the physiological and clinical differences of their cognate ligands, is the ability of GR to self-synergize (Funder, 1997). Super-additive effects are possible with this receptor when multiple HREs bind in an N-terminal dependent fashion.

In the absence of a ligand, GR and MR are complexed with heat shock proteins (hsp) in the cytoplasm, rendering both unable to translocate to the nucleus to initiate transcription (Schulman *et al*., 1994; Funder, 1997). Upon ligand binding, the GR or MR dissociates from hsp, whereupon it can then travel to the nucleus to initiate transcription of specific glucocorticoid response genes.

Through the use of knock-out mice, the functions of GR and MR have been examined (Berger *et al*., 1996). In GR knock-out mice, an 80% lethality rate among the homozygous mutants was observed at four weeks of age. The surviving homozygotes showed no abnormalities, were fertile and reached a normal age. However, they had increased serum levels of ACTH, the pituitary hormone that regulates glucocorticoid release. Immediately after birth, the mutants had acute respiratory distress due to severe lung atelectasis in part due to the impaired expression of epithelial sodium channels and synthesis of the surfactant protein. As stated previously, glucocorticoids function in gluconeogenesis, and several enzymes important in this process were reduced in livers from mutant mice. In addition, the adrenal glands were enlarged and disorganized, and increased levels of steroidogenic enzymes (P450scc and 3β-HSD) were detected.

Alternatively, MR knockout mice die around day 10 after birth (Berger *et al*., 1996). Weight loss precedes death and there is an increase in the hematocrit, believed to be due to impaired sodium retention in the kidney. Plasma renin concentration is increased by 400%, aldosterone is increased 40-fold and angiotensin II is increased 55-fold. Clearly, the ligands that bind to these receptors, as well as the factors that regulate both the ligands and receptors, are essential for life.

Another study using cultured rat embryos and an antagonist specific for MR, ZK91587, demonstrated how essential mineralocorticoid signaling is for normal development (Mirshahi *et al*., 2002). Adverse effects on total length, somite number

and embryo curvature were observed. Plus, communication between the vitelline and umbilical blood vessels in the allantoid was severely affected. Administration of aldosterone reversed these effects. Other developmental effects were observed in the limbs, optic stalk and brain formation. Similar, but less dramatic results were seen with RU26752 (anti-MR) and RU38486 (anti-GR).

The concentration of glucocorticoids can affect receptor levels in specific tissues (Erdeljan *et al*., 2001). GR was highest in the hippocampal pyramidal neurons, with appreciable amounts in glial cells. MR was detected in pyramidal neurons, with no expression in glial cells. DEX (10 and 100 nM) exposure decreased GR mRNA levels in pyramidal neuorns. However, DEX had no significant effect on MR mRNA levels. CORT also downregulated GR mRNA expression, but had no effect on MR mRNA levels. The neurotransmitter, serotonin increased GR mRNA levels; MR mRNA levels were not affected. Glial cells were not affected by any treatment.

Agonists and Antagonists to GR and MR

Because GR and MR are highly homologous to each other, it is difficult to find specific ligands that will only activate one, but not the other receptor (Raynaurd *et al*, 1980). The steroid hydroxyl groups confer specificity between receptors; DEX is most specific to GR, while the adrenal steroid, aldosterone (ALDO), is most specific to MR (Hellal-Levy *et al*., 1999). However, at varying concentrations, steroids have the ability to cross-bind with alternative receptors. Therefore, the agonists and antagonists and their respective concentrations have been carefully studied so as to define the specificity of the receptors and the ligands.

Binding data (K_d) and Skatchard analyses given below for the various compounds were determined from a standard binding assay. In brief, the competition of each synthetic or natural compound for either GR or MR was measured in high-speed supernatants or "cytosols", which were prepared from receptor-rich target organs (Raynaurd *et al*, 1980). The receptor is singled out with radiolabeled ligands, either the natural hormone or a potent synthetic agonist. Relative binding affinities (RBAs) were measured under two or three different sets of incubation conditions so as to give an accurate indication of the kinetics of the test-compound/receptor interaction.

The MR agonist, aldosterone, is the principal mineralocorticoid. It acts primarily in the adrenal gland and functions to regulate sodium balance in urine (Griffen & Ojeda, 2000). Aldosterone binds both MR (K_i=0.08 nM) and GR (K_i=63 nM), however, its affinity for MR is 1000-fold higher than GR (Rupprecht *et al*, 1993).

Table 1: Relative affinities of agonists and antagonists to the glucocorticoid and mineralocorticoid receptors. The name of the ligand is given in the left column with its dissociation constant (K_i) for both GR and MR. The reference is given in parenthesis. Multiple K_i's are reported for conflicting information.

	Affinity for GR (K_i)	**Affinity for MR (K_i)**
Aldosterone	63 nM (Rupprecht, 1993) 11.89 nM (Reul, 1990)	0.09 nM (Rupprecht, 1993) 0.20 nM (Reul, 1990)
Corticosterone	4.90 nM (Reul, 1990) 5.14 nM (K_d) (Svec, 1984)	_____ 0.05 nM (Reul, 1990)
Cortisol	15 nM (Rupprecht, 1993) 5.00 nM (Reul, 1990)	0.13 nM (Rupprecht, 1993) 0.19 nM (Reul, 1990)
Dexamethasone	1.1 nM (Rupprecht, 1993) 7.75 nM (Reul, 1990) 8.5 nM (K_d) (Svec, 1984)	1.1 nM (Rupprecht, 1993) 0.42 nM (Reul, 1990)
RU 28362	_____ 0.39 nM (Reul, 1990)	>1000 nM (Rupprecht, 1993) _____
RU 38486	2.2 nM (Rupprecht, 1993) 27.90 nM (Reul, 1990)	_____ >1000 nM (Reul, 1990)
Spironolactone	79 nM (Rupprecht, 1993) 260 nM (Reul, 1990)	5.7 nM (Rupprecht, 1993) 3.25 (Reul, 1990)
Triamcinolone Acetonide	3.4 nM (K_d) (Svec, 1984)	_____
ZK 98299	38 nM (Kd) (Zhang & Danielsen, 1995)	_____

The MR antagonist, spironolactone, is a synthetic steroid with a structure similar to aldosterone, and it competes with aldosterone for binding sites on MR (Rupprecht *et al*, 1993). Spironolactone thus inhibits sodium and water reabsorption, while sparing potassium and magnesium. It has high affinity for MR, but relatively low affinity for GR. Its K_i for GR is 79 nM, while its K_I for MR is 5.7 nM, a ten-fold difference between the two.

The GR agonist, triamcinolone acetonide (TA), is a synthetic glucocorticoid. Biopotency to bioaffinity have been compared when examining the effectiveness of glucocorticoids (Svec, 1984). Binding data and Skatchard analysis can highlight binding affinities of certain ligands towards their receptors, but it does not necessarily explain biopotency discrepancies in regards to ligand concentration. It was found that TA at a concentration of 1 nM almost completely inhibited ACTH secretion in the mouse pituitary tumor cell line, the AtT-20, but at that same concentration, binding analysis revealed that only 10-20% of the cell's receptors were occupied. Binding data ranks triamcinolone acetonide (K_d=3.4 nM) as the glucocorticoid with the highest affinity for GR, before dexamethasone (K_d=8.5 nM) and corticosterone (K_d=51 nM).

The GR antagonist, ZK98299, is a synthetic glucocorticoid known to displace progesterone from its binding sites on GR. It has strong antagonistic activities, resulting in antigestational effects on the organism. In human myometrium cells, ZK98299 binds to PR, although with lower affinity than progesterone (D'souza *et al*., 1994). The binding lasts about 6 h, and the binding sites were saturated at 4-6 nM.

The K_d values for progesterone and ZK98299 were 2.46 nM and 2.2 nM, respectively. RU38486 is also a GR antagonist; however, *in vitro* it has been shown to be a partial antagonist (Zhang *et al*., 1995). ZK98299 differs from RU486 by conformation and the substituents on the D-ring of the steroid molecule. ZK98299 is considered a complete antagonist, while RU38486 is considered a partial agonist. *In vivo*, RU38486 is a partial agonist. However, *in vitro* it is a partial or complete antagonist depending on the cell line or promoter being studied. Demonstrating this is the fact that in WCL-2 cells, it is agonistic, while in COS-7 cells, it is antagonistic.

The rodent MR displays highest affinity for binding aldosterone and corticosterone (Reul, *et al*., 1990). GR has highest affinity for synthetic glucocorticoid steroids, such as RU28362 and dexamethasone, but a lower affinity for the naturally occurring cortisol and cortisone. The dog brain model was chosen to study the binding characteristics of both MR and GR and their respective ligands. Corticosterone has the highest association rate with MR, followed by cortisol, and then aldosterone. The rank order for displacement of aldosterone binding of MR was: corticosterone > aldosterone = cortisol > dexamethasone > ZK91587 > RU27652 > spironolactone >>>RU 38486. The rank order of displacement of RU 28362 for the dog GR was: RU 28362 >> corticosterone = cortisol > dexamethasone > aldosterone > ZK 91587 > RU 26752 = RU 38486 >>> spironolactone. Because of these conclusions, aldosterone and spironolactone are best as an agonist and antagonist, respectively, for MR. Triamcinolone acetonide and ZK98299 are best as an agonist and antagonist, respectively, for GR.

Dexamethasone, although a purported agonist when compared to corticosterone, is not specific enough toward GR and was not used in this study. The dexamethasone paradox, as it has been called recently, comes down to the different *in vivo* vs. *in vitro* binding properties by MR (Reul *et al,* 2000). *In vivo*, dexamethasone seems to be an impotent agonist for kidney and brain MR, while extensive *in vitro* studies have shown dexamethasone to bind with high affinity to hippocampal MR. With these and other examples, it seems that dexamethasone's finicky binding is not ideal for this study.

It was recently investigated how ligand affinity impacts receptor mobility within the living cell (Schaaf *et al*, 2003). Through the use of yellow fluorescent protein tagged human GRα(hGRα) and fluorescence recovery after photobleaching (FRAP), it was found that hGRα exhibits decreased mobility when bound to its ligand, and that this decrease in mobility is dependent on ligand affinity towards its cognate receptor. High-affinity ligands induce a larger decrease in receptor mobility than low-affinity ligands. For instance, triamcinolone acetonide and dexamethasone (K_d < 5nM) induce a profound decrease in receptor mobility, while cortisone and ZK98299 (K_d > 10nM) have little effect on GR mobility. These data suggest that ligand binding induces a conformational change of the receptor, and that this change alters the mobility of the receptor. More so, the affinity of the ligand to its cognate receptor may determine the degree of the conformational change of the receptor during binding (Schaaf *et al*, 2003

Receptor Involvement in Relation to the Effects of Glucocorticoids on Somatotroph Differentiation

Our group has also investigated involvement of GR during chicken GH cell differentiation induced by CORT (Morpurgo *et al*., 1997). CORT, progesterone and estradiol have been shown to stimulate chicken somatotroph differentiation *in vitro* as seen in the increase in the number of plaque-forming cells as measured by RHPA. However, aldosterone had no effect in this study. The GR antagonist, RU38486 (10^{-6} M and 10^{-9} M), blocked the effects of e16 serum, CORT and progesterone on somatotroph differentiation, although an ER antagonist was unable to block the effects of CORT on somatotroph differentiation. This study demonstrates that CORT is most likely working through GR and not ER to induce somatotroph differentiation.

Similarly, pituitary cells isolated from e12 chick embryos were cultured in the presence of vehicle, CORT at 1nM, the GR type II antagonist, ZK98299 at 10 uM, and CORT and ZK98299 in combination (Bossis & Porter, 2001). Immunocytochemistry revealed that treatment with CORT significantly increased the population of somatotrophs, however, the effect was not completely blocked, although it was reduced, by the antagonist ZK98299. When this was repeated with antagonists toward both GR and MR (spironolactone), the CORT induction was completely blocked. This shows that both receptors are involved in the CORT response. Continuing this study, e12 pituitary cells were pretreated with 10 μM of

ZK98299 or RU486 for 1 h before addition of 2 nM of CORT. The cells were further cultured for 36 h and then somtatotroph differentiation was estimated by ICC. Neither of the antagonists alone could block the induction by CORT (Bossis & Porter, 2004). CORT is still able to induce somatotroph differentiation when GR is blocked, which means that CORT is able to activate MR in the absence of GR.

Aldosterone was able to induce somatotroph differentiation, lending evidence that MR is activated in this response, as well. Spironolactone, a MR specific-antagonist, was unable to block the CORT induction of somatotroph differentiation. However, the two antagonists, spironolactone and ZK98299, administered together were able to block the CORT- or aldosterone-induced somatotroph differentiation in e12 pituitary cells in culture. These results were obtained visually through GH staining in ICC. Similar results were seen using an in situ GH mRNA quantitation method developed by the same authors (Bossis & Porter, 1999). ZK98299 was able to attentuate the CORT response by >50% while spironolactone attenuated the response by 65%. The two agents in combination blocked the CORT response by >90% (Bossis & Porter, 2004).

This study demonstrates that CORT is activating both MR and GR in order to induce somatotroph differentiation in e12 chicken pituitary cells. Through western blotting, it was found that GR is present as early as e8 and persisted through e12 when the study was terminated (Bossis & Porter, 2004). The beta subunit of MR was detected in low levels on e10 and then significantly increased by e12. The alpha

subunit was first detected on e12. Dual labeling immunofluorescence on e12 pituitary cells revealed that greater than 95% of GH positive cells also expressed MR.

In the brain, MRs seem to be occupied to some degree at basal levels of circulating CORT, while GRs are mostly unoccupied (de Kloet *et al*., 1993). Coupled with the evidence that when the level of circulating CORT increases, GRs become occupied after MR saturation (McEwen *et al*., 1992), it is likely that GR modulates CORT's effects in the brain, while MR has the role of constitutive activation. Likewise, GR was expressed in almost all chick e12 pituitary cells (>95%) using a gluteraldehyde-based rapid fixation/permeabilization method, suggesting universal expression of GR in pituitary cells (Bossis & Porter, 2004). Using dual fluorescence ICC, MR was expressed in greater than 90% of e12 cultured GH-staining cells after the cells were treated with CORT for 24 h, lending further evidence that CORT activates MR preferentially and then activates GR if necessary

Conclusion

Somatotroph differentiation in the chicken pituitary gland begins around e14 but can be induced 2 days prematurely both *in vitro* and *in vivo* with the administration of CORT. CORT increases both GH mRNA and protein production, but protein synthesis of unknown protein(s) is required before induction of GH synthesis. The effects of CORT on GH secretion becomes maximal at e16 after which the increased GH production diminishes. By e20, it seems that somatotrophs are unresponsive to CORT's effects. The change in responsiveness to CORT may be due to a change in the involvement of the type of glucocorticoid receptor that CORT is working through.

In order to determine how the thyroid hormones and CORT interact to influence GH production in the pituitary during the second half of chicken embryonic development, we cultured embryonic chicken pituitary cells in the presence of CORT and T_3 as well as agonists and antagonists specific for the glucocorticoid and mineralocorticoid receptors. An ELISA was used to measure GH secreted into the cell culture medium. Through the use of dual-fluorescence immunocytochemistry for GH and MR, we were able to determine receptor expression in somatotrophs during this period of development.

Chapter 2:Somatotropin Response to Corticosterone (CORT) and the Thyroid Hormone, T_3, During Chick Embryonic Development: Involvement of Type I and Type II Glucocorticoid Receptors

Introduction

Current research conducted in our lab centers on the differentiation of somatotrophs in chickens and the extrapituitary signals that regulate this phenomenon. The chicken is a unique model for studies of anterior pituitary development because maternal interactions are not a factor as in mammals and the egg also allows for easy manipulation of the embryo for a multitude of experiments.

The glucocorticoid, corticosterone (CORT), is the extrapituitary signal that induces final somatotroph differentiation in embryonic chickens, which typically occurs on embryonic day (e) 14 (Porter *et al*., 1995a; Morpurgo *et al*., 1997; Dean *et al*., 2001). Administration of CORT induces somatotroph differentiation and a significant increase in GH secretion by cultured e12 pituitary cells after two and six days in culture, as determined by immunocytochemistry (ICC) and reverse hemolytic plaque assay (RHPA), respectively (Dean *et al*., 2001).

Pituitary cells appear to become non-responsive to CORT between e16-e20 (Porter & Dean, 2001), and endogenous CORT peaks at the time of hatching (Kalliecharan & Hall, 1974). Dispersed pituitary cells obtained from e16, e18 and e20 chick embryos were treated with CORT for 6, 12 and 18 h while subjected to RHPA. On e16 and e18, CORT increased the number of GH plaque forming cells after 18 h. On e20, CORT significantly increased the number of GH secreting cells after 12 h in the RHPA, but this response was lost after 18 h. After 24 h in the

RHPA, all ages tested were unresponsive to CORT. Results from RHPA require careful interpretation during late embryonic chick development because a significant increase in GH secretion may be missed as the number of plaque-forming cells detected approaches the absolute number of somatotrophs present.

The apparent difference in responsiveness of somatotrophs from e12 to e16-e20 to CORT was demonstrated under different experimental conditions and by varying assays, e.g. extended culture followed by ICC or acute treatment in the RHPA itself. Directly quantifying GH secretion by pituitary cells at specific intervals during the last half of embryonic development with one type of assay under one experimental condition will demonstrate more conclusively differences in somatotroph responses to CORT during development.

Somatotroph differentiation and GH secretion are also regulated by the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), where T_4 must be converted to T_3 before it has its effects, and T_3 modulates the CORT response in a bi-phasic manner (Liu et al., 2003). At low doses (0.01 nM), T_3 is slightly stimulatory alone and augments the CORT effect on GH cell differentiation by e11 pituitary cells. At higher doses (1 and 10 nM), T_3 antagonizes the CORT response. T_3 suppresses GH secretion by pituitary cells obtained from e16 embryos after 32 h in culture and this effect is lost by e18, as demonstrated by RHPA (Dean & Porter, 2001). CORT in combination with T_3 suppresses GH secretion on e16-e20 after 24 h in culture as determined by RHPA. Clearly, GH secretion induced by CORT is confounded when T_3 is present as well.

Recent evidence shows that CORT induced somatotroph differentiation involves both the type I (mineralocorticoid, MR) glucocorticoid receptor and the type II (glucocorticoid, GR) glucocorticoid receptor (Bossis & Porter, 2004). CORT induced GH cell differentiation in cultured e12 pituitary cells was only blocked when treated with both the MR specific antagonist, spironolactone, and the GR specific antagonist, ZK98299, as determined by ICC. GR is expressed in all pituitary cells at e12, and MR is co-localized with GH greater than 90% of the time in e12 somatotrophs, as shown by ICC and dual label immunofluorescence, respectively. Investigating CORT induction of somatotrophs using specific glucocorticoid receptor agonists and antagonists may elucidate the reason for the apparent change in responsiveness to CORT during chick embryonic development.

In this study, a sandwich enzyme-linked immunosorbent assay (ELISA) for chicken GH was used to quantify GH secretion in response to CORT, T_3, and GR and MR specific antagonists during the last half of chick embryonic development, in order to determine, using a unified approach, whether GH responsiveness to CORT is lost during chick embryonic development and to assess the involvement of GR and MR in this response.

Materials and Methods

Animal Use and Cell Culture

Cell culture reagents were purchased from Gibco Invitrogen (Grand Island, NY). Hormones and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. All procedures were approved by the Instituitional Animal Care and Use Committee (#R-00-51). Animals used in this study were Avian x Avian strain or Ross x Cobb strain chicken embryos purchased from Allen's Hatchery (Seaford, DE). Eggs were incubated in a humidified incubator (G.Q.F. Manufacturing, Savannah, GA) at 37.5 ^{o}C and 60% humidity. Eggs were set in the incubator such that embryos of different embryonic ages were available for dissection on the same day. Pituitaries were removed from embryonic chickens on specific days of development using a dissecting microscope and then pooled and dispersed with trypsin as previously described (Porter et al, 1995a). $2x10^{5}$ cells suspended in DMEM (Dulbecco's modified Eagle's medium, serum free) were allowed to attach for one hour in 24-well culture plates and then 0.5 mL of serum-free culture medium (SFM; a 1:1 mixture of phenol red-free Medium 199 and Ham's F12, supplemented with 0.1% BSA, 5 ug/mL bovine insulin, 5 ug/mL human transferrin, and penicillin/streptomycin) was added. The plates were incubated for 24 or 72 h in the presence of treatments: CORT, T_3 and T_4 alone and in combination ($1x10^{-9}$M).

GHRH ($1x10^{-8}$M) was added to the culture well for the final six hours of incubation to stimulate the release of GH from the cells. After that time, medium was collected from each well, centrifuged for 10 minutes at 300 G to remove any loose cells, and then the supernatants were frozen at –20 ^{o}C for later ELISA analysis.

Sandwich ELISA for chicken GH

The sandwich ELISA assay for chicken GH was conducted using the monoclonal antibody (mAb), mAb 6F5 (Houston et al, 1991) and the polyclonal chicken GH antiserum developed by Porter, 1995a. Briefly, 96-well Nunc-brand Immunosorp© plates were pre-coated with the monoclonal antibody (1 μg/mL in 50 mM Na_2CO_3, pH 9.6) overnight at 4 ^{o}C as previously described (Houston et al, 1991). All incubations were conducted in a 37 ^{o}C incubator with several PBS (phosphate buffered saline; 100 mM phosphate, 78 mM NaCl, 72 mM KCl, pH=7.4) washes between each subsequent incubation. Samples and standards diluted in 0.1 M PBS/0.1% bovine serum albumin (BSA) were added and incubated for 2 hours in a 37 ^{o}C incubator. Liquid was aspirated and the plates were rinsed 5 times. The plates were blocked (1 M PBS/0.1% BSA/1% normal goat serum (NGS)) for 30 min. Liquid was aspirated, and the plates were rinsed 3 times. A polyclonal rabbit anti-chicken GH antiserum (1:1000) was added for 90 min. Liquid was aspirated and the plates were rinsed 5 times. Goat anti-rabbit IgG biotinylated antibody (1:200) was added for 30 min. Liquid was aspirated, and the plates were rinsed 3 times. The plates were incubated with a commercially available goat anti-rabbit avidin-biotin

horseradish peroxidase (1:500) for 30 min. Liquid was aspirated and the plates were rinsed 5 times. After that time, tetra-methyl-benzidine (TMB) was diluted per the manufacturer's instructions and was added as the assay substrate. The plates were developed until all wells exhibited some colored product; the development time typically lasted for 2 to 5 minutes at room temperature. The reaction in all wells was stopped with the addition of 1 M H_2SO_4. The plates were read immediately using a Wallac© (Perkin-Elmer) plate reader with the absorbance set at 450 nm. The secondary antibody, enzyme complex (avidin-biotin horseradish peroxidase) and the developing reagents (TMB) were obtained from commercial kits (Vector Laboratories; Burlingame, CA). The average GH concentration for each treatment was determined from triplicate ELISA wells performed on triplicate cell culture wells.

Statistical Analysis

Each experiment was replicated 4 to 6 times. Statistically significant differences among treatments were determined using a mixed model ANOVA and a test of Least Squares Differences (LSD) in the SAS statistical program (SAS Inc; Cary, NC). Values were considered significant at $p \leq 0.05$. Data was transformed into the log scale to correct for normality and then back-transformed into the geometric mean. Values reported are the geometric mean and one standard error above and below the mean.

Results

Validation of Sandwich ELISA for chicken GH

The first specific aim of this project was to establish an ELISA for chicken growth hormone, which was adapted from Houston, 1991. This assay uses a monoclonal antibody against chicken GH (generously provided by B. Houston). The polyclonal GH antiserum raised in rabbit was developed and validated in our lab (Porter et al., 1995a). Because very few commercial enzyme kits are available for chicken proteins, a biotinylated goat anti-rabbit IgG was used to link the rabbit anti-chicken GH antiserum to the color-producing enzyme, a strept-avidin conjugated horseradish peroxidase. The biotin-avidin secondary detection system was used to amplify the GH signal. The substrate specific for the peroxidase is hydrogen peroxide, while the assay substrate was tetramethylbenzidine, and sulfuric acid was used to stop the reaction.

To validate an ELISA, four concepts were considered: specificity, sensitivity, recovery, and parallelism. The specificity of the assay is conferred by the monoclonal antibody, which binds only chicken GH and not bovine GH, chicken and ovine prolactin, luteinizing hormone and porcine follicle-stimulating hormone (Houston, 1991). Specificity is also conferred by the polyclonal antibody that does not bind chicken prolactin (Porter, *et al*., 1995a). The sensitivity of the assay relates to the lowest concentration in the standard curve different from the zero GH standard.

In this case, the assay cannot differentiate between 1 ng/mL and 2 ng/mL, but can differentiate between 2 ng/mL and 8 ng/mL. Therefore the sensitivity of the assay is roughly 5 ng/mL. Recovery is the amount of protein detected by the assay in contrast to the known amount. Standard curves prepared in PBS and culture medium were used for this. Cell culture medium was plated in the ELISA wells at three different amounts: 10 μL, 30 μL, and 100 μL. This also changed the amount of buffer in each well. The amount of buffer, ranging from zero percent to 100%, changed the slope and results of the standard curve (Figure 1). Even altering the concentration of phosphate in the PBS had little effect on the results. With the addition of BSA, however, the results from standard curves prepared in PBS or culture medium were nearly identical, demonstrating both recovery and noninterference by BSA. Through the use of the recombinant chicken GH standard and the secreted cellular GH, the ELISA correctly estimated the concentration of GH present, indicative of parallelism.

The coefficient of variation is a unitless measure of the variability of the data. It is determined as the variance divided by the mean of the data set. If the CV is small, then the data has low variance. The CV estimated for an ELISA is based on a known sample that is plated in every plate. In this case, a single well containing the 32 ng/mL standard was used in each plate. A replicate is defined as one ELISA assay using multiple plates was conducted on a single day. Replicates that used one, two or more than four plates were not used to calculate the CV. Preliminary trials and the first experiment did not require log transformation and thus the standard curve is

linear; all following experiments were log transformed and therefore two CV's were calculated, one for the linear data and one for the log transformed data. The inter-assay CV for the linear data is defined as the variation of the 32 ng/mL standard between all replicates (n=9) and was found to be 39.9%. The inter-assay CV for the log transformed data (n=9) and was found to be 30.4%. The intra-assay CV is defined as the variation of the 32 ng/mL standard between multiple (3-4) 96-well plates within an assay on a certain date. The intra-assay CV for the linear data (n=9) was found to be 34.0%, while the intra-assay CV for the log transformed data (n=13) was found to be 29.3%. By log transforming the ELISA data, variability was reduced and repeatability was increased. However, the assay remained variable, and greater numbers of replicate experiments were required as a result.

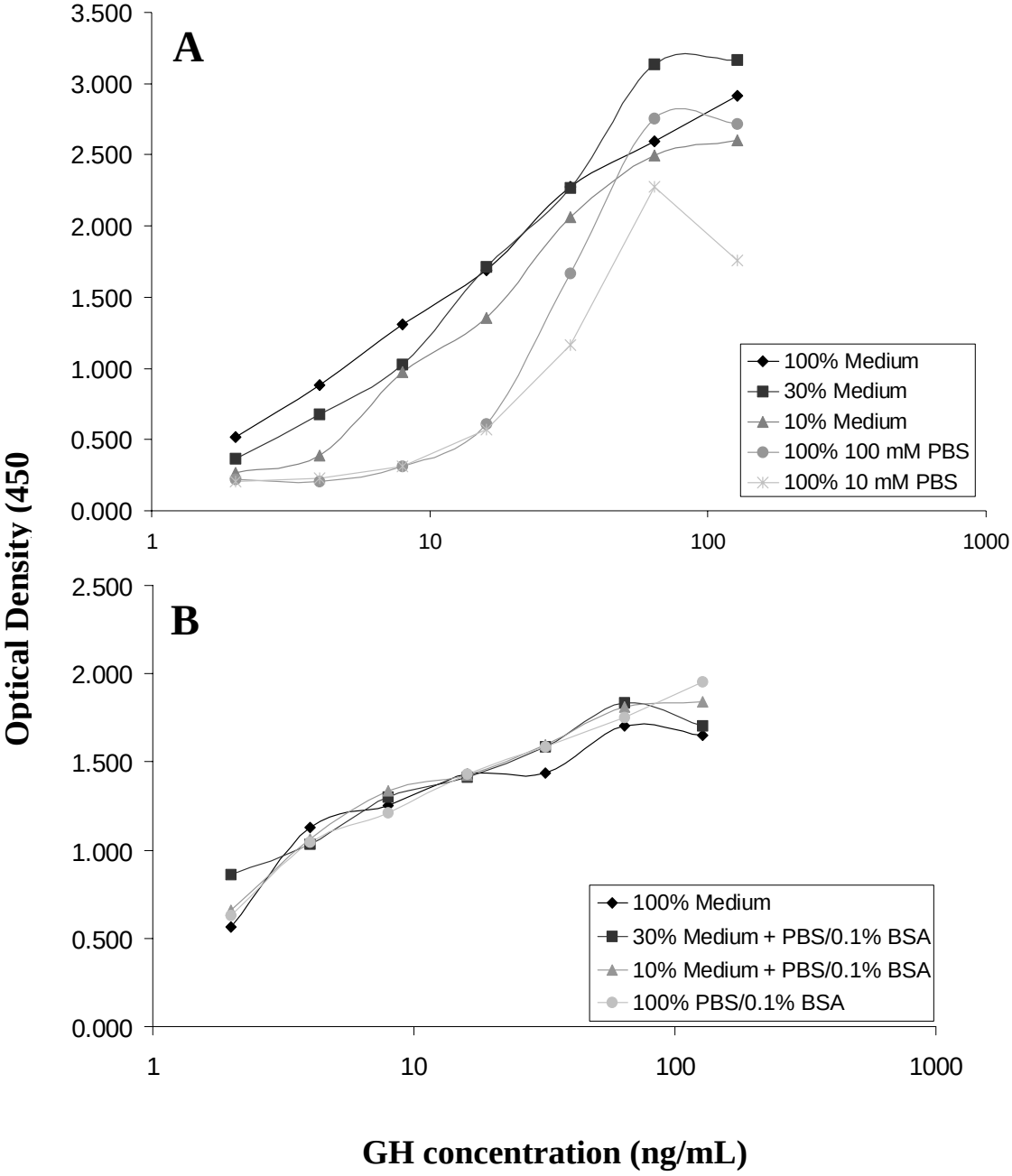

Figure 1: Standard Curve Validation. A) The results of the standard curves are dependent on the type of medium in which they are prepared. B) Addition of BSA corrects this.

Somatotropin Response to CORT: Preliminary Testing

During preliminary testing to demonstrate a difference in the CORT response between the ages of e12 and e20, a cell culture system and its parameters were established. Previous research demonstrated that protein synthesis was required for GH induction in response to CORT (Bossis & Porter, 2003) and maximum secretion was reached at 18 h (Dean et al., 1997). Because of this, e12 somatotrophs would have to be cultured for at least 18 h. The 24 h time-point was chosen for two reasons, first, it was enough time for cells to produce and secrete GH, and second, GHRH could be added at a reasonable time to induce the cells to release their stored GH. Further, the effects of GHRH on somatotrophs are maximal after 8 h and significant effects of GHRH are seen after 6 h (Dean et al., 1997). It has also been demonstrated that somatotrophs continue to secrete GH when treated with CORT and GHRH after 2 and 6 days in culture (Dean & Porter, 1999). Thus, somatotrophs could be kept in culture for up to 6 days, and a GH response to CORT could still be observed.

From preliminary testing using the sandwich ELISA, e12 cells treated with 0.5 nM of CORT were not secreting enough GH into the media at the cell density that they were plated at for the ELISA to detect differences in basal vs. CORT levels using 100 uL of culture medium as the sample. However, after 72 h in culture with the addition of the same concentration of CORT and GHRH added during the final six h in culture, the ELISA could detect GH in the cell medium. Cultured e20 cells

produced excessive amounts of GH after both 24 h and 72 h in culture, such that the medium had to be diluted (1:50) before assaying it with the ELISA.

Experiment I: Somatotropin GH secretion in response to GHRH, CORT, and triiodothyronine, T_3, during the last half of chick embryonic development

The objective of the first experiment was to characterize the response of somatotrophs to CORT and the thyroid hormones across developmental ages during the last half of development using one culture system and one assay system. Research conducted in this lab centering on the somatotroph response to CORT during development has used multiple different cell culture and assay systems to address the effects of CORT on GH secretion at different embryonic ages. Primary pituitary cell cultures have been manipulated with varying times in culture and with varying treatments to elucidate the cellular and molecular mechanisms that control somatotroph differentiation. But more importantly, RHPA, ICC, RIA and mRNA quantitation have been used extensively and although each individual assay is sound, all of them produce slightly different answers to the question. RHPA relates area of plaque produced to quantity of GH secreted. ICC shows the number of cells containing GH and not the amount of GH produced and secreted. Quantifying mRNA directly addresses the levels of the GH transcript but does not address mRNA stability, degradation, and the amount of protein being translated. RIA is the only assay, other than ELISA, that directly quantifies the amount of protein secreted. However, RIA requires the use of radioactive materials and experience. ELISA,

similar to RIA, quantifies the amount of protein without the use of radioactivity and can be completed in one day. For these reasons, the somatotroph response to CORT during the second half of embryonic development was characterized using one culture system and a single ELISA.

Avian x Avian strain fertilized eggs were placed in a 37.5 ^{o}C humidified incubator such that on the day of the experiment, there were embryos of e12, e14, e16, e18 and e20 ages. The dissected pituitaries from one age were dispersed and pooled as previously described (Porter et al., 1995a), plated in triplicate culture wells, and treated with CORT (0.5 nM) and T_3 (0.5 nM) alone and in combination. Dissection of all embryos took one hour and the entire dissection and dispersion was completed in 3.5 h. Six hours before termination of the experiment, the appropriate wells were treated with GHRH [10 nM] to stimulate the release of GH into the cell culture medium. The duration of treatment and concentration of GHRH was chosen because maximum GH secretion was observed between 4 and 8 h after treatment with GHRH (Porter *et al*., 1995). After 72 h total culture time, the cell culture medium from each well was collected, centrifuged and stored for later ELISA analysis. Prior to freezing, the e14 samples were diluted 1:3 in PBS, e16 samples were diluted 1:10 in PBS, e18 samples were diluted 1:25 in PBS and e20 samples were diluted 1:50 in PBS.

From this, it was observed that overall GH secretion increased with age (Figure 2). The mean basal level of GH secreted by cultured cells obtained from e12

pituitary cells was 64.6 ± 6.8 ng/mL (Figure 2). Addition of GHRH increased the mean GH concentration to 140.7 ± 20.0 ng/mL. However, this apparent effect of GHRH alone was not statistically significant. By e14, the mean GH secreted under basal conditions was 335 ± 67 ng/mL, however, the addition of GHRH did not increase the secretion any further with a mean of 260 ± 37 ng/mL. On e16, the mean GH concentration increased dramatically to 2220 ± 342 ng/mL, but the addition of GHRH again did not increase GH concentration (1490 ± 218 ng/mL) over basal levels. At e18, the mean GH secretion was 2926 ± 482 ng/mL under basal conditions. Again, GHRH did not increase GH secretion (1881 ± 233 ng/mL). On e20, the mean GH concentration increased further to 7041 ± 1146 ng/mL under basal conditions, and the addition of GHRH had no effect on GH secretion

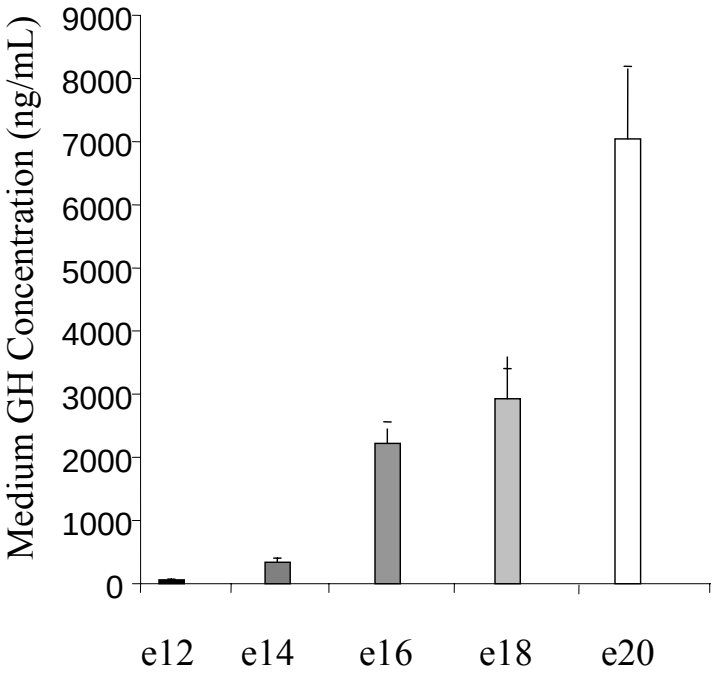

Figure 2: Basal GH secretion by cultured pituitary cells during the last half of chick embryonic development. Cells were cultured for 72 h and the cell culture medium was collected for ELISA analysis. Values are the mean ± SE of 6 replicate experiments.

(5922 ± 945 ng/mL). It is possible that GH secretion is maximal during the third quarter of embryonic development (e14-e20), which is why GHRH did not have an effect. The lack of a response to GHRH [1 nM] alone was observed previously on e16 through e20 after 18 h in culture as determined by RHPA (Dean *et al*., 1997).

CORT increased GH secretion on e12 and significantly increased it on e14, e16 and e18 in the presence of GHRH (Figure 3). CORT had no effect on GH secretion on e20. CORT treatment increased GH secretion about 1.4-fold over basal conditions both with and without GHRH on e12. On e14, CORT had no effect on GH secretion without the presence of GHRH, but produced a significant increase with the addition of GHRH as seen in the 2.1-fold increase in GH secretion over that with GHRH alone. Again, on e16, CORT treatment did not significantly increase GH secretion without the presence of GHRH. CORT treatment significantly increased GH secretion 1.6-fold with GHRH. On e18, CORT increased GH secretion, although not significantly, under basal conditions and significantly increased GH secretion with the addition of GHRH 2.5-fold. At the end of development, on e20, CORT had no effect on GH secretion with or without GHRH. The transitional age from CORT responsive to CORT non-responsive was found to be e16.4 using a regression line fitting of the entire data set. Specifically, a regression line was fitted to the basal level of GH secretion across the five ages. A second regression line was fitted to the CORT-induced level of GH secretion across the five ages and the point at which the two lines were no longer different was designated as the "non-responsive point."

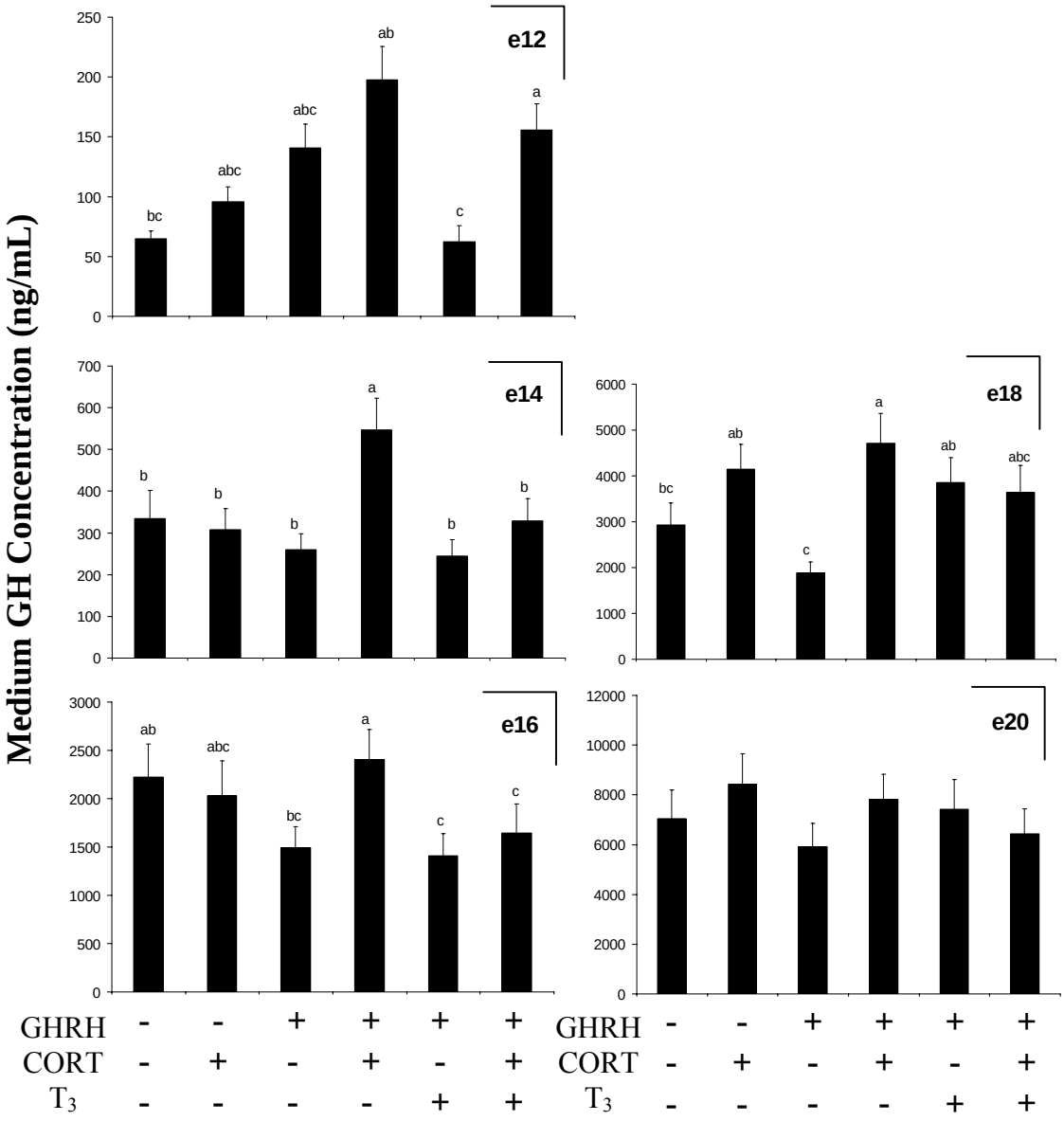

Figure 3: GH secretion by pituitary cells during the last half of embryonic development. Cells were treated with CORT and/or T3 [0.5 nM each] for 72 h and then with (+) or without (-) GHRH [10 nM] during the final six h in culture. Values are the mean ± SE of 6 replicate experiments. Values with different letters denote significant differences at $p \leq 0.05$ within ages.

T_3 at 0.5 nM had no effect on e12, e14, e16 and e20 (Figure 3). T_3 at the same concentration increased GH secretion on e18 from 1881 ± 233 ng/mL to 3852 ± 547 ng/mL. When T_3 was given in combination with CORT, it was observed that T_3 attenuated the CORT response on e14-e18. There was no effect of T_3 on the CORT response seen in e12 and e20 cells. All cells treated with T_3 were also treated with GHRH to release stored GH.

Experiment II: Determination of Glucocorticoid Receptor Involvement in the Response of Cultured Pituitary Cells to CORT and Modulation by T_3

The specific aim of experiment II was to determine which receptor (GR or MR) was used by CORT to induce GH secretion. Because the somatotroph response to CORT changes 1-4 days before hatching, some aspect of CORT induction must have changed. The receptor that CORT is activating is one obvious choice. CORT primarily binds with the type I glucocorticoid receptor, also known as the mineralocorticoid receptor (MR), but CORT is also able to bind with the type II glucocorticoid receptor (GR). It is possible that CORT preferentially binds to and activates MR from e12 through e16 and then after this time point, MR is either sequestered or not expressed and CORT activates GR instead.

In order to address this question, this experiment used antagonists and agonists of both MR and GR at or slightly above their respective effective concentration as demonstrated in relevant literature. By using these agents at the concentration of their K_d, it can be assumed that the agonists and antagonists are only activating the receptor that they have the most specificity toward. For this experiment, we chose aldosterone (ALDO) and spironolactone as a MR-specific agonist and antagonist, respectively. The GR-specific agonist and antagonist utilized were triamcinolone acetonide (TA) and ZK98299, respectively. Dexamethasone was not chosen as a GR-specific agonist because it has been demonstrated that this agent also has a high affinity for MR. Almost all synthetic and natural glucocorticoids and

mineralocorticoids are able to activate both GR and MR when administered at appropriate doses. For this reason, when choosing agonists and antagonists to use, we specifically chose agents that had at least a 10-fold lower affinity for the opposing receptor. And finally, this experiment was designed to determine what effect T_3 has on this system.

Primary pituitary cells obtained from e14 and e20 embryos were used. The cells were incubated for 72 h as before. Both CORT and T_3 were used at 1 nM, while ALDO was used at 0.5 nM. The MR specific antagonist, spironolactone, was used 10-fold higher than ALDO at 5 nM. The GR antagonist, ZK98299 was used at 5 nM, which is the same as spironolactone and also ten-fold higher than ALDO and five-fold higher than the CORT concentrations used. CORT and ALDO significantly increased GH concentration in the cell culture medium over basal conditions on e14 and had no effect on e20. Administration of TA and T_3 alone produced no significant effect on both e14 and e20. Addition of the antagonists had no effect on the CORT response on both ages. (Data not shown). As no effects of the antagonists were found, the results of this study were inconclusive and the concentration of the agonists and antagonists warranted a more thorough investigation.

Experiment III: Determining the effective concentrations of GR and MR specific agonists and antagonists

Because the initial experiment with agonists and antagonists of MR and GR did not produce easily interpretable results, the next step was to determine the effective concentrations of both the agonists and antagonists in this pituitary cell culture system using the sandwich ELISA. From these results, the concentrations of the agents used would not solely be based on relevant literature and physiological concentrations, but from actual GH responses.

This experiment used the same 72 h incubation time with GHRH being added during the final six hours of culture. The five treatments, CORT, ALDO, TA, spironolactonte, and ZK98299 were each administered at three different concentrations in three-fold increments, beginning with the concentrations used in the previous experiment and increasing the concentration from there. CORT was administered at three doses: 1 nM, 3 nM, and 9 nM. ALDO was used at 0.5 nM, 1.5 nM, and 4.5 nM. TA was initially administered at 0.5 nM, 1.5 nM, and 4.5 nM and then in subsequent replicates administered at 0.1667 nM and 13.5 nM. Spironolactone was used at 5 nM, 15 nM, and 45 nM initially and then three-fold higher again at 0.135 μM. ZK98299 was similar to spironolactone and administered at 5 nM, 15 nM, and 45 nM initially and then higher at 0.135 μM in later trials. T_3 was dropped from this experimental design because the concentration of T_3 was not under question and the experimental design became too difficult.

The main question that needed to be answered was at what concentration would ALDO elicit an increase in GH levels but still be low enough that its specific antagonist, spironolactone, would block this response when administered together. The same principle was applied to TA and ZK98299. Furthermore, concentrations of the antagonists should be low enough so as not to be stimulatory when administered alone or with CORT. The concentrations of the antagonists were also titered against CORT. Only cells from e14 embryos were used to initially determine effective concentrations.

The results show that CORT (1 nM) induced a four-fold increase of GH over basal levels (Figure 4A). There was no effect of CORT on GH secretion at 3 nM and 9 nM. The reason for this is not clear, but it may reflect involvement of two receptors that elicit different responses. ALDO did not significantly increase GH secretion at any concentration used in this experiment. Again, TA had no effect on CORT at any concentration used. All of the TA binding and affinity studies referenced used either human or rodent cells or isolated receptors and ligands in test tubes. There was no indication from the literature that TA would not elicit an increase in GH secretion in embryonic chick pituitary cells. Spironolactone, the MR antagonist, did not induce GH secretion at any concentration used (0.5, 1.5 and 13.5 nM) (Figure 4A).

The next objective was to determine the effective concentrations of spironolactone and ALDO when administered together (Figure 4B). The specific aim was to find a concentration of ALDO that would significantly increase GH secretion over basal levels and then counter that response with a concentration of

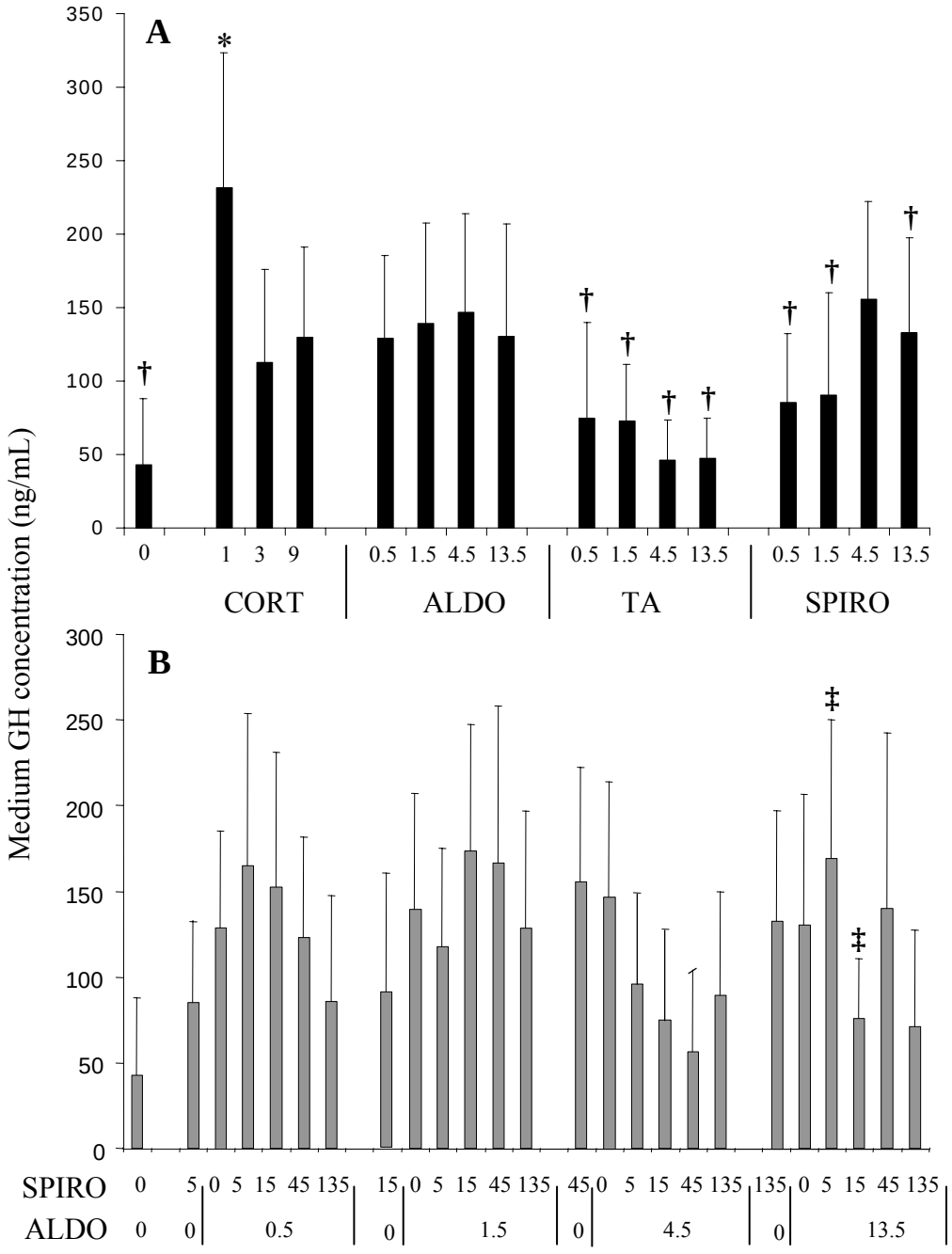

Figure 4: GH secretion by e14 anterior pituitary cells in response to (A) varying concentrations (nM) of CORT, ALDO, TA, and SPIRO. B) Assessment of effective concentrations (nM) of ALDO and SPIRO. Cells were treated with these agents for 72 h and then treated with GHRH during the final 6 h. Values are the back transformed means of log transformed data and plus one standard error of three replicate experiments. (*) denotes significant differences from basal at $p \leq 0.05$. (†) denotes significant differences from CORT (1 nM) at $p \leq 0.05$. (‡) denotes significant differences from each other at $p \leq 0.05$.

spironolactone that would block the ALDO-induced GH increase and not have any effect on GH secretion when administered alone. Cultured cells treated with ALDO at each of the four concentrations did not increase their GH secretion significantly over basal levels. Because ALDO did not elicit a GH response, it could not be determined if spironolactone at a specific concentration would block the ALDO-induced response. It has been established that an antagonist can act as an agonist if administered at high enough concentrations. The only significant differences in GH secretion were found when ALDO was administered at 13.5 nM in combination with spironolactone at 5 nM versus spironolactone at 15 nM ($p \leq 0.05$). The results of this study were inconclusive, although the most logical concentration of ALDO was determined to be 0.5 nM, because it most closely reflected the K_d from the literature and elicited a GH response over basal levels, even though the mean response at this concentration was not significant.

The effective concentration of both of the antagonists had yet to be determined. From a previous study conducted in this lab, ZK98299 was used at 10 μM to block CORT induction of GH secretion (Bossis & Porter, 2004). This time, ZK98299 would be tested at 1 μM, 0.1 μM, and 10 nM to see if any of these concentrations were high enough to be stimulatory or could affect CORT induction of GH secretion. The same 72 h total culture time was used, agonists and antagonists were added to the cell culture wells at the same time, and three complete replicates were performed.

Spironolactone alone at 5 and 15 nM, but not at 10 or 50 nM, was stimulatory to somatotrophs in culture, producing an increase in GH secretion significantly different from basal levels (Figure 5). ZK98299 alone at all three of the concentrations tested did not stimulate GH secretion. When the two antagonists were administered together at varying concentrations, some combinations did elicit a response of increased GH secretion. Spironolactone at 15 nM in combination with ZK98299 at 10 nM, 100 nM, and 1 μM elicited an increase in GH secretion that was significantly different from basal levels. However, spironolactone at the highest concentration of 50 nM in combination with ZK98299 at 10 nM failed to produce the same result. Clearly, the antagonists can act as agonists when given for 72 h at inappropriate concentrations. From these results, spironolactone should not be used above 15 nM.

In this set of experiments, ALDO at 0.5 nM did induce an increase in GH secretion (Figure 5). The addition of 5 nM of spironolactone was not able to block this response. However, the addition of 10 nM of spironolactone caused an even greater increase in GH secretion, although not significantly different from ALDO alone. ALDO with spironolactone at 15 and 50 nM produced similar results to ALDO with 5 nM of spironolactone. ZK98299 was unable to block the increase in GH secretion by ALDO, although ZK98299 at 100 nM attenuated the ALDO response significantly, but did not reduce GH levels comparable to basal levels.

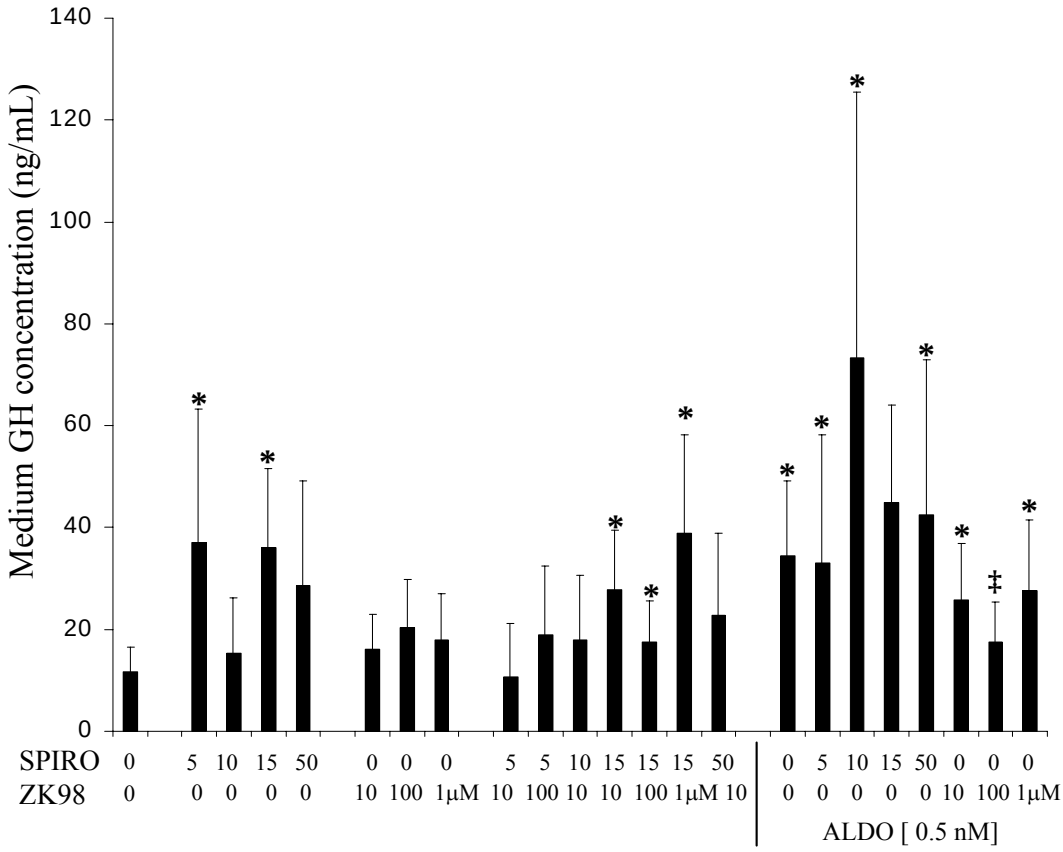

Figure 5: Medium GH concentration of e14 cultured pituitary cells after 72 h in culture with the antagonists spironolactone (SPIRO) and ZK98299 (ZK98) and aldosterone (ALDO). GHRH [10 nM] was added during the final six h in culture. All concentrations are nanomolar, unless otherwise specified. Values are the back transformed means of log transformed data and plus one standard error of three replicates. (*****) denotes significant differences from basal at $p \leq 0.05$. (‡) denotes significant differences from ALDO at $p \leq 0.05$.

The same experiment was repeated with CORT at 1 nM (Figure 6). Spironolactone alone at 1, 5, and 15 nM, but not at 1.5 nM, significantly increased GH secretion. ZK98299 alone at 10 nM, 100 nM, and 1 μM did not elicit a response. CORT produced a significant increase in GH secretion over basal levels. When the antagonists were administered with CORT, neither of them blocked the CORT induced increase in GH secretion. When the antagonists were administered together with CORT, they again did not block the CORT induced increase in GH secretion. Only with spironolactone at 1 nM, ZK98299 at 100 nM and CORT at 1 nM was there a decrease in GH secretion. This apparent decrease was not significantly different from CORT alone nor basal levels of GH secretion; it was intermediate.

The concentrations of each of the agents used in this experiment were examined closely and none of the results were conclusive. It is possible that CORT and ALDO are binding to the receptor first and the antagonists, although at the correct concentration, are unable to gain access to the receptors initially.

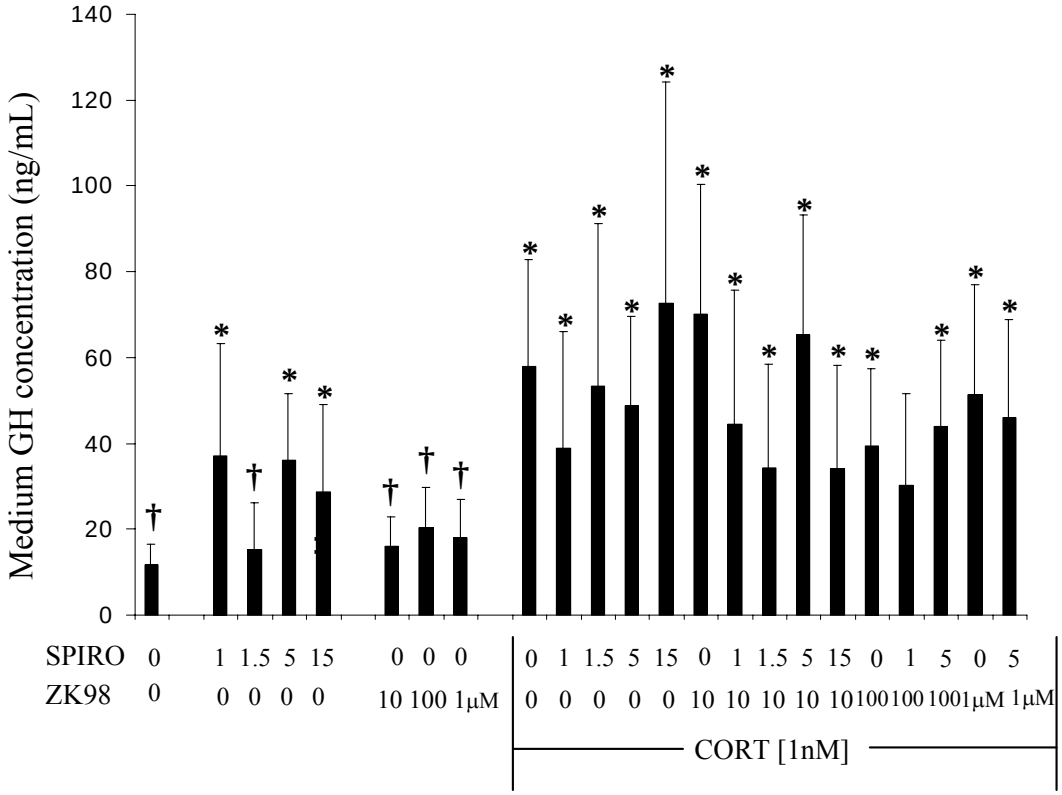

Figure 6: Medium GH concentration of e14 cultured pituitary cells after 72 h in culture in order to find effective concentrations of the antagonists SPIRO and ZK98 at suppressing the response to CORT. GHRH [10 nM] was added during the final six h in culture. All concentrations are nanomolar, unless otherwise specified. Values are the back transformed means of log transformed
data and plus one standard error of four replicates. (*) denotes significant differences
from basal at $p \leq 0.05$. (†) denotes significant differences from CORT alone at $p \leq 0.05$.

Experiment IV: Pretreatment with Antagonists to Determine Receptor Involvement during the CORT response

Clearly the concentration of the antagonists, although important, was not the only factor affecting the outcome of these experiments. If the cells were pretreated with the antagonists for 3 h, giving them time to bind to the receptors without directly competing with the agonists, would the outcome be any different? Because of the nature of antagonists, meaning that they bind in a different manner than the receptor's cognate ligand, it is possible to assert that even at the perfect concentration of both the agonist and the antagonist, the agonist will almost always displace the antagonist long enough to produce a biological response.

The objective of this experiment was to use treatment doses established by the previous two experiments, but pretreat with the antagonists to allow time for binding to receptors without directly competing with the receptor's cognate ligand. The overall culture time for this experiment was varied as well. A 72 h overall culture time may be too long of a time for the antagonists to continuously out-compete the agonists for the receptors. Previously, the 72 h culture was used over the 24 h culture time to allow the e12 cells from Avian x Avian strain embryos enough time to secrete enough GH into the cell culture medium for detection in the ELISA, even though both time points were sufficient for GH secretion. This experiment used Ross x Cobb pituitary cells, which produced more GH. Because of this, the overall culture time was varied between 72 h and 24 h. The following results (Figure 7) are from one trial

at 72 h and one trial at 24 h overall culture time. GHRH was again added at 6 h prior to the termination of each of these experiments.

Avian x Avian and Ross x Cobb are both broiler strains. Preliminary results from our lab have shown that Ross embryos have a higher abundance of somatotrophs than Avian strain embryos at the same age. Even so, they are still responsive to CORT.

Three h pretreatment with antagonists allowed time for them to bind to the receptors. A shorter overall incubation time proved effective as well. For the 24 h total culture time, the experiment was terminated 24 h after the addition of the antagonists, such that the cells were treated with the agonists for 21 total h. After 72 h, CORT increased GH secretion, and inclusion of antagonists did not suppress this increase. In the subsequent experiment, after 24 h, CORT increased GH secretion and inclusion of spironolactone alone did not suppress the CORT response. ZK98299 alone and in combination with spironolactone appeared to attenuate the CORT response. This suggests that CORT activates either GR or MR to induce GH secretion. Of course, these results should be taken lightly because each experiment was only replicated once. Even so, for subsequent experiments, the 24 h time period was adhered to strictly for all replicates.

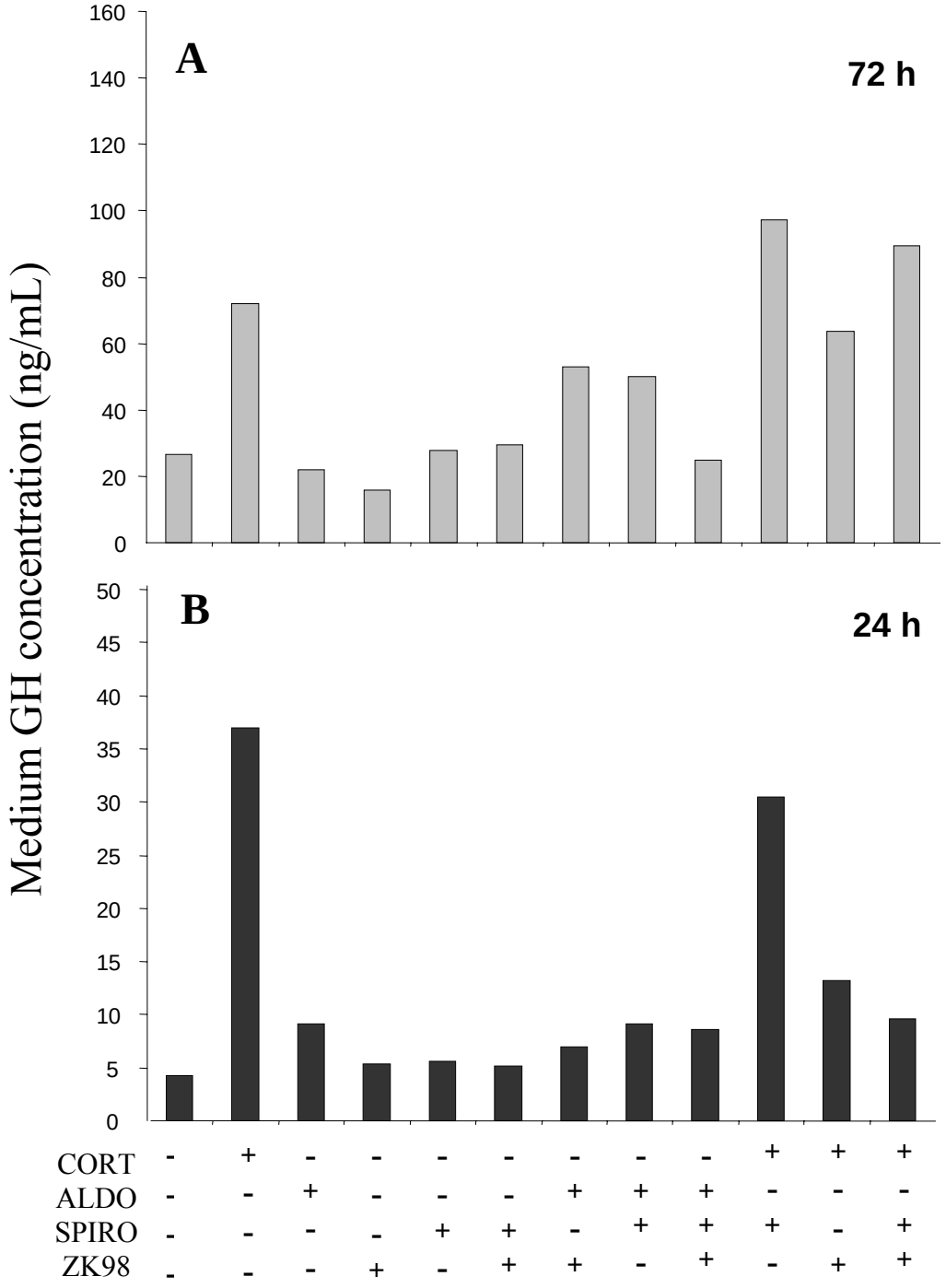

Figure 7: Effects of 3 h pretreatment with antagonists and overall culture time on GH secretion by pituitary cells obtained from e12 Ross x Ross strain embryonic chicks. A) The antagonists have no effect after 72 h in culture. B) After 24 h in culture, SPIRO still has no effect, but ZK98 and SPIRO in combination with CORT attenuates the CORT response. Values are the geometric mean of triplicate culture wells of one experiment.

Experiment V: Pretreatment with Antagonists to Determine Receptor Involvement in CORT Responsive vs. CORT Non-responsive Somatotrophs

After the previous experiments, it was found that the most effective approach was to culture the cells for 24 h total and pretreat the cells with the antagonists for three hours before the addition of the agonists. The objective of this experiment was to test whether both ZK98299 and spironolactone are required to block the CORT response on e12 and to determine if receptor involvement is a factor in the loss of responsiveness to CORT of pituitary cells late in embryonic development, i.e. e20 cells.

Ross x Cobb eggs were set in the incubator such that both e12 and e20 embryos were dissected on the same day. Cells were pretreated with spironolactone at 5 nM (low dose) and 15 nM (high dose) and or ZK98299 at 10 nM. CORT (1 nM) and ALDO at 0.5 nM (low dose) and 1.5 nM (high dose) were added to appropriate wells 3 h later. The cells were cultured for 24 h total. GHRH was added during the final 6 h of total culture time, and four replicates were conducted.

Cultured pituitary cells obtained from e12 embryos were responsive to CORT treatment as seen in the significant three-fold increase in GH secretion (6 ± 3.5 ng/mL) over basal levels (2 ± 0.95 ng/mL) (Figure 8A). ALDO at both the high and low doses also produced a significant increase in GH secretion. ALDO at the high dose produced a five-fold increase over basal levels, much higher than observed in previous experiments, although this response was more variable. The two antagonists, ZK98299 and spironolactone, at both the low and high doses, did not

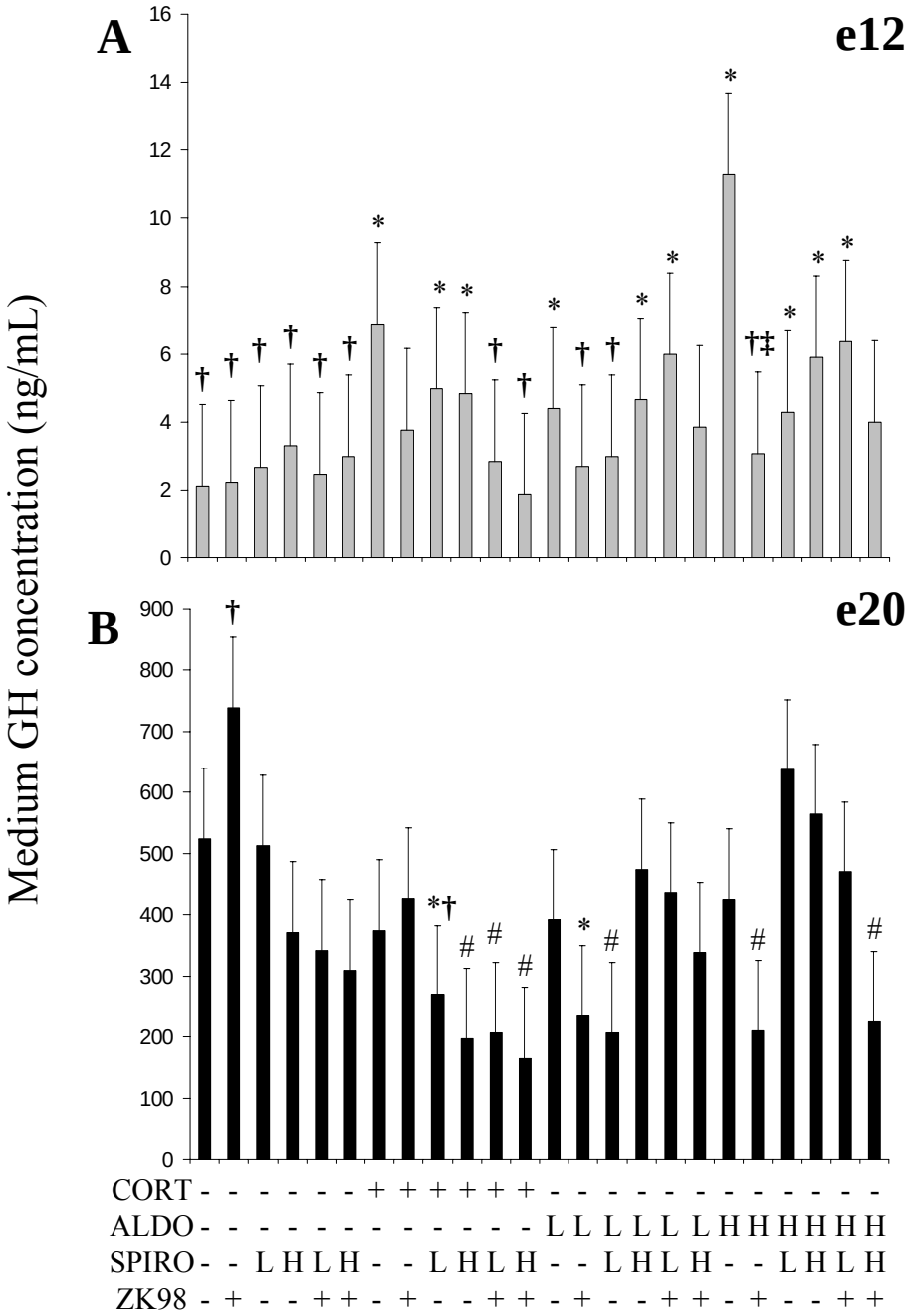

Figure 8: Determination of receptor involvement in the CORT response in GH secretion by (A) e12 and (B) e20 cultured pituitary cells. Cells were cultured for 24 h with a 3 h pretreatment with the antagonists, SPIRO and ZK98. Concentrations of treatments were as follows: CORT at 1nM, ALDO at 0.5 nM (L) and 1.5 nM (H), SPIRO at 5 nM (L) and 15 nM (H), and ZK98 at 10 nM. Values are the back transformed means of log transformed data ± pooled standard error of four replicates. (*) denotes significant differences from basal at $p \leq 0.05$. (†) denotes significant differences from CORT at $p \leq 0.05$. (‡) denotes significant differences from ALDO (H) at $p \leq 0.05$. (#) denotes significant differences from BASAL, CORT, and ALDO at $p \leq 0.05$.

stimulate GH secretion. The same result was seen when the cells were treated with the two antagonists simultaneously.

Addition of ZK98299 suppressed the ALDO induced increase at both the high and the low doses. Spironolactone at the low dose (5 nM) also significantly suppressed the low dose ALDO induced GH increase. However, SPIRO at the low dose had no effect on the high dose ALDO response. Spironolactone at the high dose had no effect on either of the doses of ALDO. ALDO was used as a positive control for spironolactone; but spironolactone was unable to block all of the ALDO induced increases in GH secretion. Furthermore, inclusion of the two antagonists with ALDO at both doses did not suppress the ALDO induced increase in GH.

From these observations, it seems that ALDO is inducing GH secretion by e12 pituitary cells through the GR receptor and not the MR receptor because ZK98299 attenuated the ALDO response, but spironolactone was ineffective. Addition of both antagonists with ALDO may cause over recruitment of receptors and GH secretion is not suppressed because of a high level of activated receptors. Alternatively, inclusion of the MR agonist and antagonist (3 compounds with the capacity to interact with MR and/or GR to some extent) may have resulted in GH stimulation through MR activation.

ZK98299 alone was unable to block the CORT-induced increase in GH secretion, and the resultant GH level was neither different from basal nor CORT levels. Spironolactone alone at both the high and low doses was unable to block the CORT-induced increase in GH secretion. However, when both the antagonists were

used together in combination with CORT, there was no increase in GH secretion, indicating that CORT could induce GH secretion through either GR or MR.

Repeating this experiment with cultured pituitary cells obtained from e20 chick embryos produced unexpected and interesting results (Figure 8B). Each of the four agents alone (CORT, ALDO, ZK98299 and spironolactone) had no effect on GH secretion. ALDO at both doses in combination with ZK98299 significantly suppressed GH secretion (235 ± 62 and 210 ± 67 ng/mL) compared to basal levels (524 ± 177 ng/mL). The low dose of ALDO in combination with the low dose of SPIRO also significantly suppressed GH secretion (207 ± 44 ng/mL). GH secretion was also suppressed significantly when ALDO at the high dose was administered in combination with spironolactone at the high dose and ZK98299. The combination of CORT and the low dose of spironolactone significantly decreased GH secretion (267 ± 83 ng/mL) compared to basal levels (524 ±177 ng/mL). When the high dose of spironolactone was used, suppression of GH secretion (196 ± 48) was significantly different from both CORT and basal levels. With the addition of ZK98299 to CORT and spironolactone similar suppressive results were observed.

In summary, CORT significantly increases GH secretion by cultured pituitary cells obtained from e12 chick embryos, but has no effect on e20. The CORT induced increase in GH secretion on e12 was effectively blocked by inclusion of both antagonists, spironolactone and ZK98299. Neither antagonist alone could block the

CORT response. On e20, inclusion of antagonists with CORT or ALDO suppressed GH secretion below basal levels.

Discussion

A sandwich ELISA for chicken GH to estimate GH secretion by cultured chick embryonic pituitary cells was modified from Houston (1991). The specificity of both the monoclonal and polyclonal antibodies was validated previously (Houston *et al*., 1991; Porter *et al*., 1995a). Further validation of the assay included estimates of sensitivity and repeatability, and demonstration of parallelism. Addition of BSA to samples and standards produced parallel standard curves, regardless of the medium in which the samples were suspended. The interassay CV estimated for this assay was 35.2%, while the intraassay CV was 31.7%. With an assay variability of over 30%, only two-fold changes or higher in GH concentration could safely be considered significantly different. Even with this variability, however, the ELISA for chicken GH has its advantages. The assay could be completed inexpensively in one day. This ELISA was able to detect differences in GH secretion by pituitary cells during the last half of chick embryonic development. From this assay, basal GH secretion by cultured pituitary cells ranges from 65 ng/mL on e12 to 7000 ng/mL on e20, with a dramatic increase on e16. These changes agree with those in endogenous levels of circulating GH during chick embryonic development (Scanes, 1987) and the ontogeny of GH-secreting cells (Porter *et al.*, 1995a).

The present results demonstrate that CORT induces GHRH stimulated GH secretion in cultured pituitary cells obtained from e12 to e18 and that this effect is lost by e20. Previous work has demonstrated that CORT can increase the number of GH

producing cells among cultured e12 pituitary cells using ICC and RHPA (Morpurgo *et al*., 1997; Dean & Porter, 1999; Bossis & Porter, 2001). In a follow-up paper, e16-e20 somtatrophs were no longer responsive to CORT after 24 h in culture in an RHPA (Porter & Dean, 2001). Estimation of secretion by RHPA requires that the cells be cultured in small amounts of medium and possibly for an extended amount of time. Although the pituitary cells obtained from embryos later in development were repeatedly unresponsive to CORT, it is possible that the unresponsiveness was due to an assay artifact. ICC, RHPA, and ELISA are inherently different in what they measure. ICC estimates GH containing cells, while RHPA demonstrates GH secreting cells. Neither of these assays quantifies the level of GH secretion, which can be done with an ELISA. Moreover, the previous findings were determined under varying experimental conditions. The present study is the first time that the loss of responsiveness to CORT by e20 pituitary cells during late chick embryonic development has been demonstrated using the same experimental and assay conditions used to show stimulatory effects on e12. GH secretion by the pituitary is at a maximum as GH peaks just prior to hatching (Scanes, 1974), which may be why CORT is unable to elicit a response over already increased GH secretion. Alternatively, the change in responsiveness may reflect differences in receptor levels, occupancy, or involvement.

Our results demonstrate that the somatotroph response to CORT is modulated by T_3 only on specific embryonic ages in development. E12 pituitary cells are unresponsive to the combination of CORT and T_3 after 3 days in culture. However,

on e14 and e16, T_3 suppresses the CORT-induced increase in GH secretion. On e16 and e18, T_3 alone increases GH secretion over basal levels and this effect is lost by e20. There was no combined effect of CORT and T_3 on e18 or e20 relative to basal and CORT treated. Other studies have demonstrated that the combined effect of CORT and T_3 is super stimulatory on e11 after three and six days in culture (Liu *et al*., 2003), but by e16, the combined effect was inhibitory to acute GH secretion (Porter & Dean, 2001). One explanation for the differing results may be that these two studies used different culture conditions and different assays to determine the effects of glucocorticoids and T_3 on GH secretion. However, it is well known that glucocorticoids and thyroid hormones interact to affect GH gene transcription in rats (Evans *et al*., 1982; Shapiro *et al*., 1978). Thyroid hormones induce GH synthesis 5- to 20-fold, and cortisol increases this response 2- to 6-fold higher in cultured rat GH1 cells (Shapiro *et al*., 1978). In rat pituitary GH3 cells, the effects of T_3 on the thyroid hormone receptor (TR) and GR were studied in parallel with T_3 and dexamethasone (DEX) treatment on GH gene transcription (Williams *et al*., 1991). T_3 and DEX increased GH gene transcription, both alone and in combination, and this effect was still observed while TR and GR mRNA levels were reduced. This is evidence that receptor presence is not the only factor regulating GH gene transcription. TR is also part of the nuclear/steroid receptor superfamily. Studies have shown that TR can form heterodimers with the Vitamin D receptor (Kliewer *et al*., 1992; Zhang *et al*., 1992). It is possible that heterodimerization of TR and GR/MR is one modulator of GH gene transcription. Whether the responses involve direct interactions of nuclear

receptors or not, the present results demonstrate that the nature of the GH response to CORT and T_3 changes during development.

It was hypothesized that the change in responsiveness to CORT from e12 to e20 may be due to a change in glucocorticoid receptor involvement. Cultured e14 pituitary cells were treated for 72 h with GR and MR specific antagonists, ZK98299 and spironolactone, respectively. The antagonists exhibited unexpected paradoxical effects on GH secretion after this amount of time in culture. By definition, antagonists should not decrease basal activities, but only suppress a given response. Spironolactone at most concentrations tested paradoxically stimulated GH secretion. ZK98299 alone was not stimulatory. The two antagonists in combination with an agonist were also stimulatory. After reducing the culture time to 24 h and pretreating e12 pituitary cells with the antagonists, the same concentrations of the antagonists (5 nM and 10 nM) were no longer stimulatory, and when given in combination with an agonist suppressed GH secretion relative to the agonist alone. It appears that the concentration of the antagonist was not in excess, rather the amount of time the cells were incubated with the antagonists produced the paradoxical effects. No evidence of these unexpected effects of the antagonists could be found in the literature. Pituitary cells and cell lines have been treated with agonists, such as CORT, dexamethasone and triamcinolone acetonide, for as long as 6 days in culture without producing anomalous effects (Sve, 1984; Dean *et al*., 1999). The paradoxical effects of long term exposure to these glucocorticoid receptor specific antagonists in chick embryonic pituitary cells warrants a more thorough investigation.

Treatment with a GR specific antagonist, ZK98299, or a MR specific antagonist, spironolactone alone, did not abolish the CORT mediated induction of GH secretion by e12 pituitary cells after 24 h in culture, but treatment with both antagonists completely blocked the effect of CORT on GH. This suggests that CORT can exert its actions through either GR or MR to induce GH secretion. If GR is blocked, then MR is activated to affect GH secretion, and likewise for MR. Induction of GH by both MR and GR has also been demonstrated by estimating the number of GH containing cells using ICC when treated with these same receptor specific antagonists (Bossis & Porter, 2004). GR is expressed in almost every pituitary cell type on e12, and using dual-labeling immunofluorescence, e12 GH containing cells co-expressed MR greater than 95% of the time (Bossis & Porter, 2004). GR and GH were colocalized in 98% of cells in the rat anterior pituitary as well (Ozawa *et al*., 1999). It is believed that in the adult brain MRs are occupied with basal levels of CORT most of the time and that GRs are mostly unoccupied (de Kloet *et al*., 1993). Further, when the circulating level of CORT increases, GRs are activated after MR saturation in neuronal tissue (McEwen *et al*., 1992). Thus, GH secretion in response to low levels of CORT may be mediated primarily through MR, but when CORT levels rise, GRs become activated as well. This is further supported by the finding that GR and MR are known to heterodimerize, and upon heterodimerization, DNA specific binding is greatly increased (Trapp *et al*., 1994). Because the two receptors are expressed in the majority of pituitary somatotrophs on e12, heterodimerization of receptors may play an important role in the hypothalamic-pituitary-adrenal axis,

while heterodimerization in peripheral tissues, such as the liver where MR expression is low, is less critical (Funder, 1993).

It appears that receptor involvement does not change by e20 in chick embryonic pituitary cells, however, enigmatic effects were observed. On e12, treatment with either MR or GR antagonists alone did not block CORT induced GH secretion, and inclusion of both antagonists was required to block CORT induction of GH secretion. On e20, inclusion of antagonists alone did not suppress GH secretion; rather the addition of CORT and ALDO with antagonists was GH suppressive. These findings of inhibitory effects of MR and GR selective antagonists on e12 and e20 suggest that both receptors are expressed in somatotrophs throughout chick embryonic development. That said, the suppression of basal GH secretion by MR and GR antagonists with an agonist on e20 is enigmatic. However, at e20, prior to hatching, endogenous CORT is high. One hypothesis is that endogenous CORT may have been carried over from the intact embryo through to the primary pituitary culture, where GR and/or MR were already affecting GH gene transcription. High levels of endogenous CORT may also explain the unresponsiveness of e20 pituitary cells to CORT in culture; they may have already been maximally stimulated by endogenous glucocorticoids. This hypothesis is unlikely because inclusion of both antagonists without an agonist present should have suppressed GH secretion and this was not observed. Moreover, during the dispersion process, cells are bathed in CORT-free medium, diluting the concentration of any endogenous CORT. Further, the cells are washed three times in fresh medium that would wash away any

endogenous CORT. Activation of the glucocorticoid receptors by appropriate ligands causes the translocation of the receptor from the cytoplasm to the nucleus, where binding of the receptor ligand complex initiates gene transcription. The inclusion of the antagonists with an agonist, thereby activating both GR and MR, may have caused a conformational change in the receptors resulting in the suppression of normally expressed gene products, while increasing transcription of gene products yet to be determined.

In summary, glucocorticoids increase GH secretion during the last half of chick embryonic development until just prior to hatching, when the cells are no longer responsive. Thyroid hormones modulate this effect. Both type I (MR) and type II (GR) glucocorticoid receptors are involved in this response.

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