Validation of a Culture System for Porcine Pituitary Cells: Effects of Growth Hormone-Releasing Factor and(or) Somatostatin on Growth Hormone Secretion

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ABSTRACT: The goal of the study was to establish the age-related responses of cultured porcine pituitary cells to growth hormone-releasing factor (GRF) and(or) somatostatin (SRIF). A culture system for dispersed porcine pituitary cells was validated. Pituitaries from female pigs of various ages (90 or 110 d of gestation, newborn, 3, 6, or 24 mo old) were enzymatically dispersed with collagenase and neuraminidase, plated (200,000 cells/well), and cultured for 3 d. Plated cells were then subjected to a 4-h challenge with increasing concentrations of GRF to $10^{-11}$ to $10^{-8}$ M, SRIF to $10^{-9}$ to $10^{-6}$ M, or $10^{-8}$ M of each peptide with increasing concentrations of the other. Culture media were collected and assayed for growth hormone (GH). Pituitaries were pooled so that there were four replicates per age, and treatments were assigned to quadruplicate wells. Concentrations of GH in control wells (basal GH) were maximal at 110 d of gestation and decreased thereafter ($P < .01$) with increasing age of swine. All peptide combinations affected the GH response ($P < .05$) at all ages studied, yet GRF was more potent than SRIF in eliciting a response. Age had an effect ($P < .05$) on the GH response to any of the treatments; younger pigs (90, 110 d of gestation and newborns) had a greater response ($P < .05$) than older pigs (3, 6, and 24 mo), whereas 6- and 24-mo-old pigs responded similarly in all cases ($P > .1$). Developmental changes in the response to GRF given alone occurred during fetal life (between 90 and 110 d, $P < .01$) and between 3 and 6 mo of age ($P < .01$), whereas changes in the response to SRIF took place between 110 d of gestation and birth ($P < .01$). It can be concluded that GRF and SRIF elicit GH responses in cultured porcine pituitary cells from pigs of 90 d of gestation until 24 mo of age, but the magnitude of the response is greatly affected by age.

Key Words: Porcine, Pituitary, Tissue Culture, Somatotropin, Somatostatin, GHRF

Introduction

Pituitary secretion of growth hormone (GH) is under the dual control of hypothalamic GH-releasing factor (GRF; Guillemin et al., 1982) and somatostatin (SRIF; Brazeau et al., 1973). Secretion of GH by the pituitary undergoes dramatic changes with age. Fetal swine harbor much greater concentrations of GH than do neonates or market pigs (Klindt and Stone, 1984). The GH response to GRF is also altered by age (Dubreuil et al., 1987; Farmer et al., 1990); younger pigs show a greater response. The involvement of GRF and SRIF in the regulation of pituitary secretion of GH at various stages of development is still unclear. The use of in vitro culture of pituitary cells permits studies of the role of the pituitary on the age-related changes in serum GH concentrations. Such data were obtained in sheep and humans and the authors suggested that pituitary insensitivity to the suppressant effects of SRIF may explain in part elevated serum GH concentrations in fetuses (Goodyer et al., 1987; Silverman et al., 1989). However, Blanchard et al. (1988) did not report an age-related sensitivity to SRIF in ovine fetuses and lambs, yet GRF and SRIF were both active from as early as 70 d of gestation and SRIF could block the GRF-induced GH release from 100 d of gestation up to the prepubertal period. It is only recently (Heiman et al., 1990) that an in vitro culture technique was used to study GH regulation at
the pituitary level in swine. It was established that anterior pituitary cells from barrows and gilts respond to GRF in a dose-dependent manner and that somatotrophs of growing peripubertal gilts are more responsive to GRF stimulation than are cells from their castrated male siblings (Heiman et al., 1990). The objectives of the present study were to validate a sensitive in vitro culture system for porcine pituitary cells and to determine the ontogenetic changes in the pituitary response to GRF and(or) SRIF in swine.

Materials and Methods

Animals

Yorkshire × Landrace female pigs were used in the study. The effect of age on pituitary function was assessed using 90 d (n = 39) and 110 d (n = 27) fetuses as well as newborn pigs (< 24 h postpartum, n = 29), 3- (n = 14) and 6-mo-old (n = 8) prepubertal pigs, and multiparous sows that were approximately 2 yr of age (n = 5). Newborn pigs were allowed to nurse before collection of the pituitaries and the 2-yr-old sows were killed at 90 or 110 d of gestation. Animals from each age group were killed (stunning and exsanguination) and pituitaries were pooled to form four replicates per age group. The pituitary glands were removed aseptically within 5 min of death, were placed in 4°C sterile collection buffer, and were kept on ice until dispersion. Maximum time elapsed from collection to plating was 6 h.

Preparation of Buffers and Media

Collection buffer (HEPES buffer) was composed of 2.5 mg/mL of bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO), 10 μg/mL of deoxyribonuclease (D-5025; Sigma Chemical), 152 M NaCl, 5 mM KCl, .7 mM Na2HPO4, 23 mM HEPES (H-0763; Sigma Chemical), and 11 mM glucose in distilled, deionized water. Collagenase and neuraminidase buffer were made with collection buffer. Collagenase buffer contained 2 mg/mL of collagenase (CLS 4196) (Worthington, Miles Canada, Etobicoke, ON) and 1 mg/mL of dispase (165-859) (Boehringer, Landrace, Lincoln Park, NJ). Cultures were kept at 37°C in a 5% CO2 atmosphere for 3 d. On d 3, cells were rinsed twice with testing media and 1 mL of the supernatant was removed and the cells were rinsed twice with 20 mL of collection buffer (37°C), resuspended in 15 to 20 mL of collagenase buffer, and transferred into a sterile, siliconized, 25-mL spinner flask (Belco Glass, Vineland, NJ). The tissue was then agitated in a 37°C incubator (5% CO2) at medium speed (approximately 300 oscillations per minute) for a maximum of 1.5 h (until very few tissue chunks were present) and was triturated with a 10-mL serological pipette for 5 min every 30 min. The suspended cell mixture was centrifuged at 200 × g (22°C) for 5 min. The supernatant was removed and the cells were resuspended in 20 mL of neuraminidase buffer before being incubated in a water bath at 37°C for 20 min. The suspension was then centrifuged (200 × g, 5 min) and the supernatant removed. Cells were washed and triturated three times with 20 mL of plating media (room temperature), centrifuged (200 × g, 5 min), and then resuspended in a known volume of plating media. Viable cells (88.5 ± .87% of total cells) were determined using the Trypan blue exclusion method and 2 × 105 viable cells per well were plated in Falcon 24-well plates (Catalog no. 3847; Becton Dickinson Labware, Lincoln Park, NJ). Cultures were kept at 37°C in a 5% CO2 atmosphere for 3 d. On d 3, cells were rinsed twice with testing media and 1 mL of the appropriate test media (containing various concentrations of GRF and(or) SRIF) was added to each well. Cells were incubated for 4 h and the media were collected into 1.5-mL microcentrifuge polypropylene tubes (Fisher Scientific, Ottawa, ON), which were then centrifuged for 5 min at 200 × g (22°C). The supernatant was frozen at −20°C until it was assayed for GH.

Cell Dispersion and Culture

The anterior lobe of the pituitary was dissected from the posterior lobe and was placed in Hanks' Balanced Salts (Catalog No. 1387; Sigma Chemical; 4°C) containing 200 μg/mL of streptomycin and 200 IU/mL of penicillin for a minimum of 5 min. The anterior pituitaries were then minced with two scalpel blades into 1-mm cubes. The minced tissue (approximately 3 mL) was rinsed twice with 20 mL of collection buffer (37°C), resuspended in 15 to 20 mL of collagenase buffer, and transferred into a sterile, siliconized, 25-mL spinner flask (Belco Glass, Vineland, NJ). The tissue was then agitated in a 37°C incubator (5% CO2) at medium speed (approximately 300 oscillations per minute) for a maximum of 1.5 h (until very few tissue chunks were present) and was triturated with a 10-mL serological pipette for 5 min every 30 min. The suspended cell mixture was centrifuged at 200 × g (22°C) for 5 min. The supernatant was removed and the cells were resuspended in 20 mL of neuraminidase buffer before being incubated in a water bath at 37°C for 20 min. The suspension was then centrifuged (200 × g, 5 min) and the supernatant removed. Cells were washed and triturated three times with 20 mL of plating media (room temperature), centrifuged (200 × g, 5 min), and then resuspended in a known volume of plating media. Viable cells (88.5 ± .87% of total cells) were determined using the Trypan blue exclusion method and 2 × 105 viable cells per well were plated in Falcon 24-well plates (Catalog no. 3847; Becton Dickinson Labware, Lincoln Park, NJ). Cultures were kept at 37°C in a 5% CO2 atmosphere for 3 d. On d 3, cells were rinsed twice with testing media and 1 mL of the appropriate test media (containing various concentrations of GRF and(or) SRIF) was added to each well. Cells were incubated for 4 h and the media were collected into 1.5-mL microcentrifuge polypropylene tubes (Fisher Scientific, Ottawa, ON), which were then centrifuged for 5 min at 200 × g (22°C). The supernatant was frozen at −20°C until it was assayed for GH.
**Peptides**

Somatostatin-14 and hGRF(1–29)NH₂ were synthesized and purified as previously described (Merrifield, 1963; Brazeau et al., 1973; Guillemin et al., 1982). Stock solutions of GRF were prepared at concentrations of 5 mg/mL; GRF was dissolved in 520 μL of 1 N HCl and the volume was adjusted by adding 480 μL of 1 N NaOH. The appropriate amount was then added to testing media. Stock solutions of SRIF were prepared in 2 N acetic acid at concentrations of 1 mg/mL and added to testing media in appropriate quantities.

**Growth Hormone Assay**

Concentrations of GH in culture media and in pituitary homogenates were determined by a validated RIA as previously described (Dubreuil et al., 1990). The first antibody (G. DesCotes, Sanofi, Montpellier, France) showed no crossreactivity with 1 pg/mL of thyrotropin, luteinizing hormone, follicle-stimulating hormone, or 5 μg/mL of prolactin. The intra- and interassay CV were 4.09 and 3.74%, respectively. Sensitivity of the assay was .2 ng/mL.

**Statistical Analyses**

Four replicates of each age were done, and in each replicate four wells were subjected to each of the peptide combinations. Statistics were performed with the GLM procedure of SAS (1982) and GH values were subjected to the log transformation to eliminate heterogeneity of variance. The main factor age was tested on the GH values for the control wells. A global model that included age, replicate (age), treatment, and dose (treatment) as main effects with age × treatment and age × dose (treatment) as interactions was performed on mean GH values. Because of significant interactions, separate analyses of variance were done for each treatment and each age using orthogonal contrasts to separate the age or treatment effects, whenever appropriate. The global model (without the main effect dose) as well as analyses per treatment and per age were run on the differences in GH concentrations (DIFF) between the control well and 10⁻⁸ M GRF, 10⁻⁸ M SRIF, 10⁻⁸ M GRF plus 10⁻⁶ M SRIF, or 10⁻⁸ M SRIF plus 10⁻⁸ M GRF. Statistical differences between GH values in control wells and in wells containing the lowest concentrations of each peptide (10⁻¹¹ M GRF or 10⁻⁹ M SRIF) were also assessed. The higher concentration of SRIF (10⁻⁶ M) was used when comparing the GRF plus SRIF combination with controls because SRIF was less potent than GRF in eliciting a GH response; the goal was to determine whether the addition of the second peptide would inhibit at any point the effect of the first one (which was always included at a fixed quantity).

**Results**

Response to a GRF challenge was improved when plating media was DMEM-F10 rather than DMEM-F10, with 2.7% FBS rather than 10% FBS, and with both insulin and sodium selenite instead of with either one alone. The number of cells per well could not be increased to 400,000 and the incubation time with GRF could be decreased to 3 h but not to 2 h.

Basal GH concentrations (control wells) were highest in 100-d-old fetuses and decreased thereafter (P < .01) with increasing age of swine (Figure 1). Pituitary cells from control wells were sonicated and there was an age effect (P < .001) on the concentration of GH in the homogenates; younger pigs (90 or 110 d of gestation and newborns) had greater concentrations (least squares means = 7,829 vs 2,461 ng/mL, SEM = 1,067, P < .001) than older pigs (3, 6, and 24 mo). Mean GH concentrations in culture media from each dose of each treatment at all ages studied are illustrated in Figures 2 to 5. The global analysis on the DIFF revealed highly significant age and treatment effects; however, the age × treatment interaction was also highly significant. The analysis per treatment showed an age effect on the mean GH response to all treatments (P ≤ .01). It is of interest to note that 6- and 24-mo-old pigs always responded similarly (P > .1) to the treatments.

The global analysis on the DIFF revealed highly significant age and treatment effects; however, the age × treatment interaction was also highly significant. Specific treatment effects on the DIFF are noted in Table 1. The differences between the control value and 10⁻⁸ M of GRF were always positive; when significant,
Figure 2. Effect of growth hormone-releasing factor (GRF) on the growth hormone (GH) response of pituitary cultures from female pigs of various ages. The SEM are 226, 298, 246, 222, 39, and 133 for the lines representing the youngest to the oldest animals, respectively.

Differences between controls and $10^{-8}$ M SRIF (SRIF fixed) plus $10^{-8}$ M GRF were also positive. In the other two cases ($10^{-8}$ M SRIF alone or GRF fixed [$10^{-8}$ M] plus $10^{-8}$ M SRIF) significant differences were negative. The lowest concentration of GRF (10$^{-11}$ M) increased GH concentrations ($P = .06$) at all ages except 6 mo, whereas $10^{-9}$ M SRIF had no effect on GH ($P > .1$) at all ages.

Discussion

Validation of a technique for in vitro culture of enzymatically dispersed porcine pituitary cells allows a better understanding of the specific factors controlling GH secretion at the pituitary level. Such a technique was previously used in rats (Brazeau et al., 1982; Heiman et al., 1985), humans (Goodyer et al., 1987), and sheep (Blanchard et al., 1988; Silverman et al., 1989) and was recently described (Elsaesser et al., 1988; Heiman et al., 1990) for porcine pituitary cells. The presently described technique differs from the previous ones mainly in terms of enzymes used for dispersal of cells, plating and testing media, and time of incubation in plating media. Results obtained with the present technique demonstrate its great sensitivity to GRF; $10^{-11}$ M GRF induced significant changes in GH response.

The age-related changes observed for GH concentrations in control wells reflect the ontogenetic development of GH levels reported for swine in vivo (Klindt and Stone, 1984); values were highest in fetuses and decreased thereafter to their lowest point in 2-yr-old sows. Basal GH release from ovine pituitary cells was also reported to decrease gradually as a function of age (Blanchard et al., 1988). As was seen in the present experiment, GRF stimulated and SRIF inhibited GH release in a dose-dependent manner at various stages of development in sheep (Blanchard et al., 1988). In one study the maximal responses to GRF or SRIF were not directly related to age (Blanchard et al., 1988), whereas in another study, the GH response to SRIF was affected by age, and was significantly
Table 1. Effect of growth hormone-releasing factor (GRF) and/or somatostatin (SRIF) on growth hormone concentrations in pituitary cultures from female pigs of various ages

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>GRF 10⁻⁸ M</th>
<th>SRIF 10⁻⁸ M + GRF 10⁻⁸ M</th>
<th>GRF 10⁻⁸ M</th>
<th>SRIF 10⁻⁸ M + GRF 10⁻⁸ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 d of Gestation</td>
<td>417***b</td>
<td>231***</td>
<td>49*</td>
<td>40**</td>
</tr>
<tr>
<td>110 d of Gestation</td>
<td>376***</td>
<td>243***</td>
<td>61*</td>
<td>41*</td>
</tr>
<tr>
<td>Newborn</td>
<td>385***</td>
<td>175*</td>
<td>73</td>
<td>60</td>
</tr>
<tr>
<td>3 mo</td>
<td>465***</td>
<td>321***</td>
<td>77</td>
<td>69</td>
</tr>
<tr>
<td>6 mo</td>
<td>261***</td>
<td>168</td>
<td>121</td>
<td>93</td>
</tr>
<tr>
<td>24 mo</td>
<td>255**</td>
<td>175†</td>
<td>78</td>
<td>50</td>
</tr>
</tbody>
</table>

Numbers represent percentage of difference between control well and given molarities of GRF and/or SRIF.

For GRF: P < .001; for SRIF: *P < .05; **P < .01; ***P < .001; when no superscript is present, P > .1.

Figure 5. Effect of somatostatin (10⁻⁸ M) plus increasing amounts of growth hormone-releasing factor (GRF) on the growth hormone (GH) response of pituitary cultures from female pigs of various ages. The SEM are 136, 207, 236, 194, 34, and 83 for the lines representing the youngest to the oldest animals, respectively.

Greater in neonatal than in fetal (122 to 140 d gestation) ovine pituitary cells (Silverman et al., 1989). Arbona et al. (1990) recently reported that in swine, secretion of GH by individual pituitary cells is affected by age and sex but not by selection for growth. Anterior pituitary cells from neonatal pigs (3 to 5 d) secreted more GH after a GRF challenge than did pituitaries from 3-mo-old pigs (Arbona et al., 1990). Our data corroborate those findings. A previous in vivo study in swine also demonstrated that the GH response to GRF develops with fetal age (from 66 to 110 d) and is maximal at 110 d of gestation (Farmer et al., 1992). Our present results are in agreement with these findings because GRF stimulated GH secretion at both 90 and 110 d of gestation, yet the difference in GH response between 90- and 110-d fetuses was not as apparent with the in vitro technique. This suggests that a factor other than pituitary sensitivity to GRF may have hindered in vivo GH response to GRF in 88-d fetuses.

As far as SRIF is concerned, the present experiment showed a linear decrease in GH values after SRIF administration to both 90- and 110-d fetal pituitary cells. This is in agreement with findings in vivo in which a reduction in GH concentrations was seen in late-gestation fetuses receiving a pharmacological dose of SRIF (Spencer et al., 1985), yet the response was variable and no response was seen when a lesser dose of SRIF (50 µg/kg) was injected (Farmer et al., 1992). Recent results demonstrated that insulin-like growth factor I (IGF-I) may be involved in regulating GH levels in fetal swine. It was suggested that the high circulating levels of GH in the porcine fetus may result from a lack of feedback of IGF-I and that this effect is not mediated via an immediate hypersecretion of hypothalamic SRIF (Spencer et al., 1991). Although SRIF is present in the pig fetus in late gestation, endogenous SRIF levels would not be involved in GH regulation at this stage of development (Spencer et al., 1991). Similarly, in vitro studies in sheep showed that GRF and SRIF are both active from as early as 70 d of gestation in the ovine fetus and that there is an increasing effect of IGF-I on basal and GRF-stimulated GH release during fetal development (Blanchard et al., 1988).

Even though the GH response to SRIF was not as large as that to GRF, the inhibitory effect of SRIF on the GRF-induced GH response was more pronounced than the response to SRIF alone in terms of the percentage decrease. Silverman et al. (1989) also noted that in ovine fetal and neonatal pituitary cells SRIF significantly inhibited the GRF-induced GH secretion, whereas the inhibition to SRIF alone was either not or only barely significant. Their results differ from ours, however, because they reported that
in neonates the response of cells exposed to GRF plus SRIF was 41% (± 8%) of the response of cells exposed to only GRF, whereas this number was 63% (± 3%) for fetal cells. This difference might be because GRF induced a slightly greater GH secretory response in ovine neonatal than in fetal pituitary cells (Silverman et al., 1989). Age-related differences in response to SRIF or GRF have not been compared in older animals of other species. Results from the present experiment establish that no developmental changes occur in the pituitary sensitivity to GRF or SRIF after pigs reach 6 mo of age. This is most likely an age effect because the maintain GH secretion at control levels. Results from the present study also show that with given alone, at least 100 times more SRIF than GRF is needed to establish that no developmental changes occur in the 24-mo-old (gestating) animals were very different. Conversely, significant differences in the GH response to GRF take place between 3 and 6 mo.

Goodyer et al. (1987) stated that 100 times more SRIF than GRF must be added to human pituitary cultures to block the GRF stimulatory effect and maintain GH secretion at control levels. Results from the present study also show that with given alone, at least 100 times more SRIF than GRF is needed to elicit a GH response. Such was also the case when the peptides were given in combination. The inhibitory effect of SRIF on the GRF-induced GH response is affected by age in human fetal pituitaries (Goodyer et al., 1987) and in fetal and postnatal ovine pituitaries (Blanchard et al., 1988; Silverman et al., 1989). However, a discrepancy occurs between results in sheep when assessing whether the inhibition is greater in fetal or in neonatal pituitary cells. The present results would suggest that this inhibition is greater in fetal cells and decreases with age, thereby contradicting the earlier report in sheep stating that pituitary insensitivity to the suppressant effects of SRIF may explain in part elevated serum GH concentrations in the fetus (Silverman et al., 1989). It seems more probable that endogenous GH levels in the porcine fetus are not mainly dependent on the sensitivity of the somatotrophs to GRF and SRIF but are related to the production of GRF, SRIF, and other controlling factors such as IGF-I. Another explanation might be a shift in somatotroph populations as a function of age. The lower basal GH and GH response to GRF seen at older ages in vivo (Dubreuil et al., 1987; Farmer et al., 1990) could likely be related to a decreased sensitivity of the somatotrophs to GRF. One can also not exclude the possible involvement of IGF-I in controlling GH secretion at the pituitary level.

Implications

A porcine pituitary cell culture system was developed in which hormonal effects were expressed. Specifically, growth hormone-releasing factor stimulated and somatostatin inhibited growth hormone release by anterior pituitary cells in a dose-related manner. Growth hormone-releasing factor was more potent than somatostatin in eliciting growth hormone responses. Responses to the two peptides were age-dependent and decreased as the animals got older. These findings may ultimately allow more effective manipulation of animal performance via regulation of growth hormone secretion.

Literature Cited


GROWTH HORMONE SECRETION BY PITUITARY CELLS