Ontogeny of Glycosylated and Nonglycosylated Forms of Prolactin and Growth Hormone in Porcine Pituitary during Fetal Life (43094)

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Abstract. **Although prolactin (PRL) and growth hormone (GH) were long considered to be nonglycoprotein hormones of the pituitary, glycosylated forms of these hormones have nevertheless been discovered in recent years. We determined the ontogeny of glycosylated and nonglycosylated PRL and GH during the fetal life of the pig, an animal in whose pituitary the glycosylated variant of PRL has been found in high (40%) concentrations. Swine fetuses of both sexes from lean and obese animals of Duroc × Yorkshire crosses were examined at 60, 75, 90, and 105 days of age. No appreciable differences related to sex or phenotype were noted in any of the parameters measured; therefore, data for all animals within an age group were combined. Such averages revealed considerable amounts of GH in the fetal pituitary as early as 60 days of age, whereas PRL, although detectable by radioimmunoassay and immunoblotting at all ages tested, was not present in significant amounts until 105 days of age. From its first appearance, however, almost 70% of the PRL synthesized in the fetal pituitary was of the glycosylated type. In contrast to PRL, both the glycosylated and nonglycosylated forms of GH showed a steady rate of increase throughout the observation period. The immunoblotting analyses also revealed in the fetal pituitary several intensely staining 8- to 12-kDa PRL-immunoreactive peptides of unknown identity. The occurrence of significantly greater concentrations of glycosylated PRL than of non-glycosylated PRL in the fetal pituitary during late gestation offers new possibilities for the role of this hormone in the development of the swine fetus.** [P.S.E.B.M. 1990, Vol 194]

In the growing animal, growth hormone (GH) is the major somatotrophic hormone of the pituitary (1, 2). To a lesser extent, prolactin (PRL) has also been found to exert a direct effect on body growth (3, 4). Although there are reports associating both of these hormones with fetal growth and development (5, 6), their precise roles in this important developmental process remain to be delineated. This is particularly so since a wide array of heterogeneity in the molecular structure has recently been uncovered (7, 8). Among the many structural modifications, glycosylation has come to be recognized as a major posttranslational transformation for these hormones, particularly for PRL, although significant proportions of both hormones have been found to link with carbohydrate moieties (9, 10). Among all species tested thus far, the concentration of glycosylated (G)-PRL is highest in the porcine pituitary, constituting nearly 40% of total glandular content (11, 12). Therefore, we decided to follow the ontogeny of the glycosylated variants of PRL and GH in porcine pituitary during fetal life, and to compare it with that of the nonglycosylated form.

Materials and Methods

Animals. Lean and obese pregnant sows (second parity), raised and bred in the swine facilities at the USDA Meat Animal Research Center, Clay Center, Nebraska, represent descendants developed from Duroc obese × Yorkshire obese crosses and from Duroc lean

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× Yorkshire lean crosses. Originally, the obese swine were genetically selected solely on the basis of thickness of the subcutaneous adipose tissue (backfat) (13). These sows were housed in an indoor confinement facility with a photoperiod consisting of 14 hr of light per day, and an environmental temperature of 15.5–18°C. Water was provided *ad libitum*, and a single meal provided daily consisted of 1.6 kg of 13% crude protein diet during 0–80 days of gestation, and 2.5 kg thereafter. They were sacrificed by "captive bolt" device at 60, 75, 90, or 105 days of gestation and the fetal pituitary glands were recovered. For the data herein, a total of 128 male and 130 female fetuses from 30 pregnant sows were used.

Tissues. Whole pituitary glands, obtained at the time of slaughter, were separated according to the sex of the fetus, and pooled for each litter. The glands were frozen in liquid nitrogen and stored at −70°C unit extracted for GH and PRL. For extraction, the pooled pituitary tissue for each sex from each litter was homogenized in Laemmli's (14) sample buffer containing 5% 2-mercaptoethanol, in the ratio of 20 mg/ml, heated in a boiling water bath for 3 min, and centrifuged at $1000g_{av}$ for 30 min. The supernatant, termed the original extract, was stored as such, or after dilution, at −20°C.

Gel Electrophoresis. The pituitary extract was electrophoresed in 1.5-mm thick, 12-cm long slab gels of 12% acrylamide, using the buffer system of Laemmli (14). A 25-μl sample of the appropriately diluted supernatant was applied to the gel to load 0.1- or 0.5-mg Eq tissue. The gels were either stained with Coomassie brilliant blue R-250 and destained by diffusion in 10% methanol and 10% acetic acid mixture, or were electroblotted onto nitrocellulose (NTC) filter papers for immunostaining, as described below.

Measurement of PRL and G-PRL by Immunoblotting. The NTC filters containing the proteins from the pituitary extracts were immunostained by the Western blotting method described by Burnett (15). The filters were reacted with a 1:1000 dilution of our anti-human PRL serum (VLS 1) for 18 hr at room temperature, followed by reaction with ^{125}I-labeled Protein A (~150,000 cpm/ml). The anti-human PRL serum instead of an anti-porcine PRL serum was used because the former is a well-characterized antiserum (16, 17) that strongly crossreacts with porcine PRL (16, 18). Furthermore, the currently available antiserum to porcine PRL, which we used in the radioimmunoassay (RIA), is a goat antiserum, and Protein A, which is used to visualize the immunoreactive bands in this test, does not crossreact with goat γ-globulins very well. The NTC filter was then exposed to x-ray film for autoradiography. PRL and G-PRL bands were identified on the basis of relative mobility (R_f) by concurrently running, at several concentrations (5, 10, and 20 ng), a standard porcine PRL preparation (USDA-pPRL B-1) which contained both glycosylated and nonglycosylated forms in an approximately 40:60 ratio (18). Areas of the NTC filter containing the PRL and G-PRL bands were cut out and counted in a gamma counter. Quantitative estimates of the amount of PRL and G-PRL in the pituitary extracts were obtained by extrapolating the counts to the respective standard curves constructed from the standard preparation (18).

Immunostaining for GH. Some of the NTC filters containing the pituitary proteins were reacted with a 1:1000 dilution of a monkey anti-murine GH serum (19) that crossreacts with porcine GH; they were then processed as described above in order to detect the GH-immunoreactive bands.

Measurement of GH by Dye Elution. The amount of protein in the GH band was determined by the method of Ball (20). The GH band from the dye-stained gels was excised, and the dye was eluted and spectrophotometrically quantitated, using bovine serum albumin as the standard.

Measurement of G-GH by Lectin-Binding RIA. G-GH in pituitary extracts was measured with a lectin-binding RIA described recently (21). ^{125}I-Labeled anti-GH IgG, isolated from our highly specific monkey antiserum to murine GH (19) that strongly crossreacts with porcine GH, was used as the probe. The original pituitary extract was diluted 400-fold with 1% bovine serum albumin-containing phosphosaline buffer (pH 7.4) and a 400-μl aliquot of this was pipetted in duplicate into plastic tubes coated with 0.5 ml of a 1% solution of concanavalin A for the assay. All samples were analyzed in a single assay.

RIA for Porcine PRL. Total immunoreactive PRL in pituitary extracts was measured with a homologous RIA, using a commercially obtained goat anti-porcine PRL serum (Research Products International Corp., Mount Prospect, IL). The details of the assay procedure are described elsewhere (22). The original pituitary extract was diluted 20,000-fold with 1% bovine serum albumin-containing phosphosaline buffer (pH 7.4) and a 10- or 25-μl aliquot of this was used for the assay, in duplicate. All samples were analyzed in a single assay. USDA-pPRL I-1 was used as the standard. The minimum sensitivity of the assay was 20 pg; the intrassay coefficient of variation was 5.8%.

Statistical Analyses. Statistical significance of the data was evaluated by analysis of variance and by Duncan's new multiple range test.

Results

Figure 1 presents the electrophoretic migration patterns for GH, PRL, and other pituitary proteins in the porcine fetus. The GH band was present as early as 60 days of age and increased in concentration with time. The PRL band was undetectable by dye staining at 60,

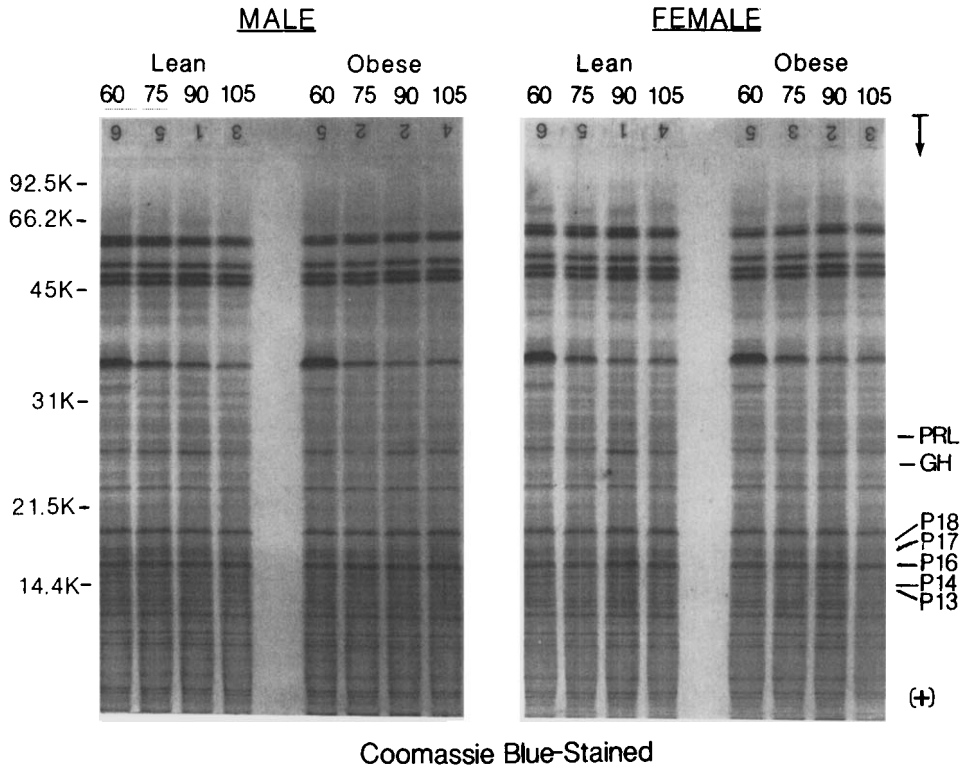

Figure 1. Electrophoretic analysis of fetal porcine pituitary showing GH, PRL, and other pituitary proteins, as revealed by Coomassie blue staining. Pooled pituitary extract equivalent to 0.5 mg of tissue from each gestational age group was electrophoresed in separate lanes. Numbers at the top of the lanes represent the days of fetal age. Numbers toward the bottom of the lanes indicate the number of pituitary pools that were combined within each age group for this electrophoresis. Numbers on the left indicate the positions of the molecular weight marker proteins. The conspicuous bands labeled as P13, P14, and so forth, on the right represent novel PRL- and GH-related peptides.

75, or 90 days of age, but became visible in all groups at 105 days. The G-PRL was not identifiable as a discrete band by dye staining at any age examined, including 105 days, because of generally weaker binding of glycoproteins with Coomassie blue. The bands labeled at P13, P14, P16, P17, and P18 represent recently reported GH- and PRL-related pituitary secretory proteins (23, 24). Not much is known about their physiologic significance (25, 26), and their role in fetal growth remains unexplored.

Figures 2 and 3 present results of the immunoblotting analyses with PRL and GH antisera. The GH antiserum elicited a single band in all pituitaries (Fig. 3). The PRL antiserum, on the other hand, elicited several PRL-immunoreactive bands of high and low molecular weight (M_r), three of which migrated in the area of M_r of 25,000–30,000. The slowest of the three, labeled as G-PRL, has been shown to be a glycoprotein by concanavalin A-Sepharose chromatography and *N*-glycanase digestion (27), and to be PRL by tyrosine peptide mapping (12). The band ahead of it was the M_r of 23,000 main PRL, and the third band is believed to be recently described "21K" variant form of PRL (28). Particularly remarkable was the prevalence of three or four PRL-immunoreactive bands of low M_r (8–12 kDa), which reacted strongly with human PRL antibodies. These bands may represent fragments of porcine PRL, although in some pituitaries the intensity of these bands was greater than that of the main PRL. A few bands of high molecular mass (>30 kDa) were also present, whose identities remain unknown. Some of them may be polymers of PRL, while others could represent PRL covalently bound to other proteins (29). The main PRL band was detectable by immunoblotting at all ages examined, in contrast to dye-staining analysis (Fig. 1), which revealed it only at 105 days of age. The G-PRL band was readily apparent in all cases at 90 days of age, but in some groups it could be seen as early as 60 days of age.

Figure 4 presents a plot of the radioactivity in the PRL and G-PRL bands of the standard preparation (USDA-pPRL B-1) versus dose, as observed in the immunoblotting analysis. The two components are present in an approximately 40:60 ratio in the USDA preparation (12, 18); G-PRL therefore would constitute 2, 4, and 8 ng and non-G-PRL, 3, 6, and 12 ng protein, respectively, at the three doses of the standard used. Both proteins produced linear dose-response curves in the 2–12 ng range, and thus the standard curves were considered suitable for estimating the amount of these hormones in the fetal pituitary. The slope of the G-PRL curve was less than that of the non-G-PRL, sug-

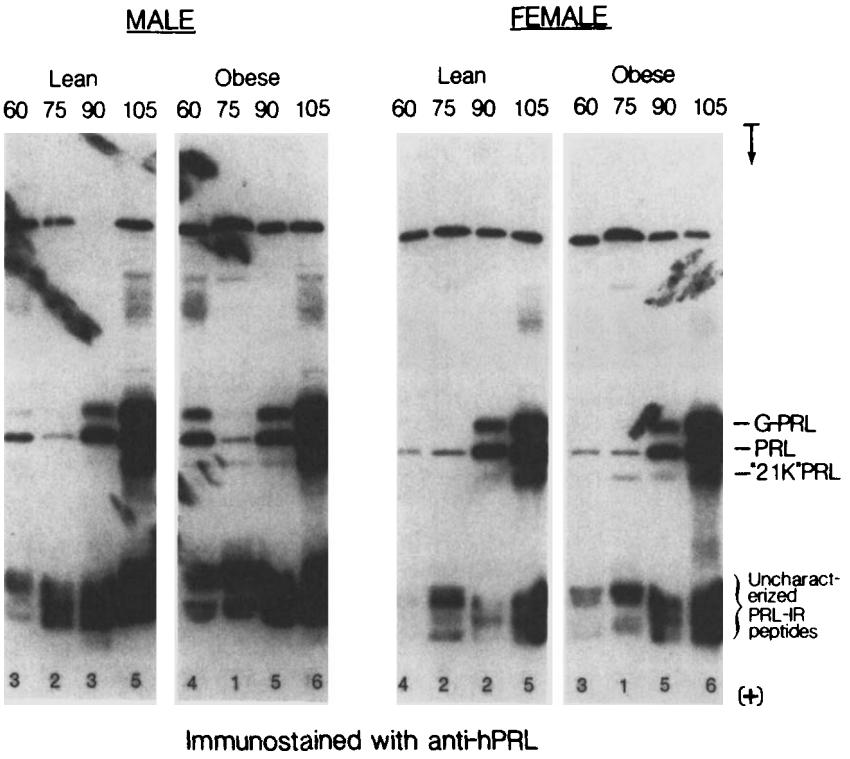

Figure 2. Western blot analysis of fetal porcine pituitary extract using an antihuman PRL serum (VLS 1) as a probe. The antiserum was used at a 1:1000 dilution. Pooled pituitary extract equivalent to 0.5 mg of tissue from each gestational age group was electrophoresed in separate lanes. Numbers at the top of the lanes represent the days of fetal age. Numbers toward the bottom of the lanes indicate the number of pituitary pools that were combined within each age groups for this illustration. The autoradiogram showed PRL, G-PRL, and several other PRL-immunoreactive (PRL-IR) bands. The identities of bands labeled as uncharacterized PRL-immunoreactive peptides are unknown.

gesting a lower (32%) crossreactivity for G-PRL with the antibody used.

Quantitative data thus calculated for the pituitary concentrations of non-G-PRL and G-PRL, for GH as estimated by the dye-elution method (20), and for G-GH as measured by lectin-binding RIA (21) are presented in Table 1. No statistical differences between male and female, or lean and obese fetuses, were noted for any of the parameters measured, and therefore the results for all animals within an age group are presented as combined averages.

PRL concentrations averaged very low in 60-day-old fetal pituitaries—less than 15 ng/mg of pituitary tissue—for both the glycosylated and nonglycosylated types (Table 1). Concentrations of both forms of PRL did not change significantly at 75 or 90 days ($P > 0.05$); however, after 90 days of fetal life, concentrations of both forms of PRL began to rise rapidly, resulting in the accumulation of several hundred nanograms of the hormone by 105 days. After 90 days, G-PRL was the major PRL form in the pituitary and registered a remarkable 2-fold higher concentration at 105 days than non-G-PRL ($P < 0.05$). Measurement by RIA, which may detect different immunoreactive forms of PRL to various degrees, displayed similar trends (Table 1), although the absolute values were somewhat lower than those measured by immunoblotting, perhaps because of the different antisera used in the two assays (30).

In contrast to PRL, GH was present in substantial amounts from the earliest testing interval, averaging over 0.70 μg/mg of tissue at 60 days of age (Table 1). Its concentration did not change significantly between 60 and 75 days ($P > 0.05$), but it increased modestly (66%) between 75 and 90 days ($P < 0.05$) and markedly (163%) between 90 and 105 days ($P < 0.05$). Changes in G-GH followed an essentially similar pattern.

Discussion

This study represents the first attempt to look at the expression of the glycosylated variants of PRL and GH in the developing embryo. The results show that the accumulation of GH in the porcine fetal pituitary begins much earlier than that of PRL; high concentrations of GH were present as early as 60 days of age, whereas PRL appeared as a distinct discernible band in dye-stained gels only at 105 days, toward the end of gestation, which averages around 114 days in the pig. Measurement of these hormones in the plasma of porcine fetuses by Klindt and Stone (31) had shown a comparable pattern for GH, but had revealed two elevations for circulating PRL—one between Days 40 and 50 and the other after Day 90. Our findings of extremely low (<20 ng/mg) concentrations of PRL in the fetal

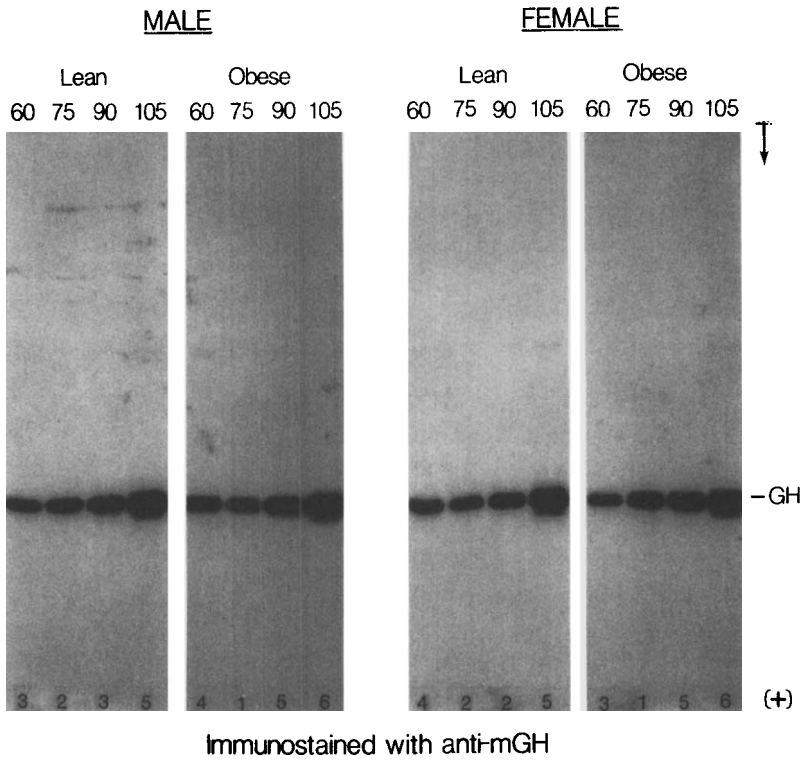

Figure 3. Western blot analysis of fetal porcine pituitary extract using a monkey anti-murine GH serum as probe. The antiserum was used at a 1:1000 dilution. Pooled pituitary extract equivalent to 0.1 mg of tissue from each gestational age group was electrophoresed in separate lanes. Numbers at the top of the lanes represent the days of fetal age. Numbers toward the bottom of the lanes indicate the number of pituitary pools that were combined within each age group for this analysis. The autoradiogram showed a single prominent GH-immunoreactive band in most cases.

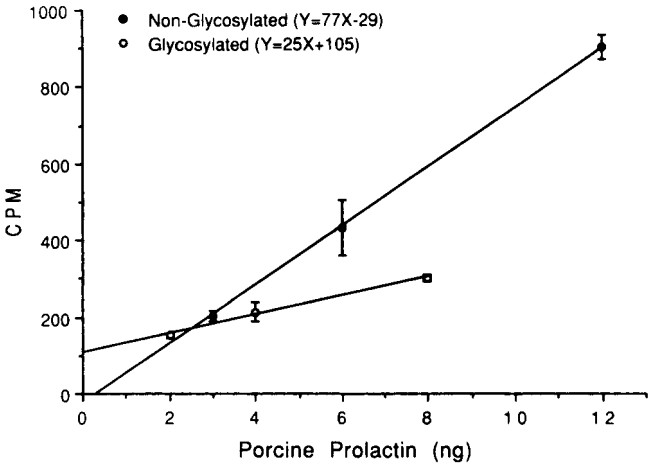

Figure 4. A plot of radioactivity in the G-PRL and PRL bands of the standard porcine PRL preparation (USDA-pPRL B-1) versus dose, showing linear dose-response relationships for the two forms in the Western blot analysis. Each point is the average of 3 to 4 replicate determinations. Vertical lines around the points represent standard errors of the means.

pituitary both at 60 and 75 days suggest that the first peak may originate from extra-fetal sources or may be an artifact of measurement. In the ovine fetus, lactotrophs were first detected by electron microscopy at about 75 days of age (32), which falls around the middle of gestation in that species (average gestation = 148 days). In humans also, lactotrophs were reported to first appear at ~112 days of gestation versus 70 days for somatotrophs (33). Thus, it is apparent from these results that fetal GH is available to fetal tissues from a much earlier age and for a much longer duration than is fetal PRL in other species as well as in the pig.

After 90 days, PRL accumulation in the porcine fetal pituitary accelerated and exceeded the rate of increase in GH accumulation. The delayed start of PRL synthesis relative to GH observed in this study is similar to the secretion patterns of these hormones observed in ovine (34) and human (35, 36) fetuses. For the first time, however, our results revealed that the PRL molecule synthesized by the fetal pituitary after 90 days was predominantly of the glycosylated type: there was more than two times as much G-PRL in the fetal pituitary at 105 days of age as there was non-G-PRL. Although we did not measure G-PRL in fetal plasma in the present study, our earlier work (18) showed G-PRL to be dominant over non-G-PRL in the plasma of pigs for 2 months after birth. Thus, if increased pituitary concentration can be equated with increased secretion, the fetal and neonatal porcine pituitary seems to secrete G-PRL to a greater extent than it does non-G-PRL.

The greater secretion of G-PRL during fetal life

Table I. Concentrations of Glycosylated and Nonglycosylated PRL and GH in Porcine Pituitary during Fetal Life[a]

Fetal age (days)	No. of pools	Total RIA PRL (ng/mg)	Non-G-PRL (ng/mg)	G-PRL (ng/mg)	GH (μg/mg)	G-GH (cpm/10 μg)
60	14	6.2 ± 2.0a	11.9 ± 2.8a	10.1 ± 2.7a	0.72 ± 0.07a	1316 ± 89a
75	6	2.6 ± 0.4a	7.3 ± 0.5a	6.3 ± 1.0a	0.97 ± 0.18a	1347 ± 136a
90	15	44.6 ± 4.7a	45.5 ± 5.3b	38.1 ± 4.3a	1.61 ± 0.13b	2001 ± 157b
105	22	489.0 ± 57.0b	205.9 ± 10.9c	455.6 ± 31.7b	4.24 ± 0.21c	3828 ± 139c

[a] Values given are mean ± SE. Statistical comparisons shown are for the data within a column; means with common letters are statistically not different from one another ($P > 0.05$).

and early infancy may be related to the kinetics of secretion of the two forms of the hormone. By pulse-chase experiments, Pellegrini *et al.* (37) found a higher secretion rate for G-PRL than for non-G-PRL in human prolactinoma cells, possibly through a constitutive pathway, while non-G-PRL secretion involved a storage step. In the fetus, the pituitary gland is still developing and thus the storage mechanisms may not be fully functional.

Besides G-PRL, our immunoblotting experiments detected in the porcine fetal pituitary tissue a number of proteins of small M_r (8–12 kDa) that crossreacted with human PRL antibodies. The degree of staining of these bands in fetal pituitary tissue was startlingly high. We have not seen such intensely staining low M_r bands in pituitaries of adult pigs (12). The nature of these PRL-immunoreactive pituitary peptides remains to be explored.

Existence of G-GH was demonstrated first in the human pituitary (10) and later in the murine (21, 38) and chicken (39) pituitaries. Although Western blotting did not reveal a G-GH band because of limits of sensitivity of the method, the lectin-binding assay detected G-GH in the porcine fetal pituitary, which underscores the frequency of its occurrence. We did not find a difference in the rate of accumulation of glycosylated and nonglycosylated forms of GH in the fetal pituitary, however. Since the accumulation of GH in the fetal pituitary had already begun long before 60 days, it is not possible to determine from our results whether there was a preferential synthesis of one form over the other during the earlier stages of adenohypophyseal development.

In the current study, no differences in the pituitary concentrations of either PRL or GH were observed between lean and obese fetuses at the ages measured. We (12) have previously reported significantly greater concentrations of pituitary, but not plasma, glycosylated and nonglycosylated PRL in *mature* obese than in lean barrows (castrated male pigs). The discrepant findings between the two studies suggest that the obesity-related differences appear later during the development of the animal, although observations of the previous study pertained to castrated pigs.

Plasma GH concentrations in fetuses of obese pigs at 110 days of gestation have been reported to be lower than those in fetuses from lean pigs (40–42). However, in those reports, consistent with our findings of the present study, differences in plasma GH were not present at 100 days of gestation (40).

What is the physiologic significance of the apparently greater synthesis of G-PRL by the fetal (and neonatal) porcine pituitary? Glycosylation has been reported to selectively alter some of the biologic activities of PRL, such as pigeon crop sac stimulation (9, 11), casein synthesis in mouse mammary gland explants (43), mitogenesis of Nb2 rat lymphoma cells (43, 44), and *in vitro* insulin-like effects on adipose tissue of hypophysectomized rats (45). However, its effects on somatotrophic and osmoregulatory actions of PRL (46) have not been evaluated. In general, glycosylation of proteins is known to reduce their clearance from circulation (47), thereby potentially enhancing their biologic effects. Furthermore, variations in the nature of glycosylation of PRL have been reported (48), one form displaying greater biologic activity than the other. Could greater secretion of G-PRL in the fetal and neonatal pig play a role in growth and osmoregulation during this period? The findings reported herein and the abundance of G-PRL in the porcine pituitary should facilitate a direct testing of these possibilities.

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