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Ontogeny of Ovine Fetal Liver and Kidney Plasma Membrane Insulin Receptors and Fetal Growth (42220)

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Abstract. This investigation was performed to define certain characteristics of insulin–receptor interaction during the last 2 months of gestation in fetal sheep liver and kidney. Twenty-one sheep carrying a total of 46 fetuses were sacrificed at various gestational ages from 94 days to term; fetal and maternal livers and kidneys were analyzed by a radioreceptor assay for insulin binding characteristics. Specific binding of insulin to partially purified ovine fetal liver and kidney plasma membranes increased as gestation approached term, at which time specific binding was two- to fourfold greater to fetal than to maternal tissues. Associated with increased specific binding were late gestational increases in affinity of insulin for receptors in both fetal liver and kidney and an earlier increase in insulin receptor concentration in fetal kidney. These observations in fetal sheep liver and kidney are similar to reported observations in other species. However, the increase in specific binding of insulin to male fetal liver membranes was exponential; in contrast, there was no apparent increase in specific binding to female fetal liver membranes during the gestational interval surveyed. Both the weights and the vertebral column lengths of these fetuses were shown by multivariate analysis to be significantly affected by the interaction between specific binding of insulin and fetal sex. However, in 30 additional sheep fetuses we observed no difference between male and female fetuses in the increase with time in liver glycogen content. The lack of sex difference in this postreceptor event is consonant with the demonstrated dissociation between liver insulin receptors and glycogen synthesis in the late fetal rat. Our observations suggest that late gestational differences between male and female sheep fetuses in insulin specific binding to liver and, possibly, to other tissues such as cartilage, muscle, and/or fat, that are coupled to postreceptor events may account for differences in fetal growth between the sexes.

Insulin plays an important role in mammalian developmental biology. It has been implicated as a major influence on fetal growth (1–5), development (6, 7), and metabolism (8–10). Thus, information regarding the characteristics of the interaction between insulin and its receptors during fetal life is important knowledge to obtain. There is evidence that the specific binding of insulin to certain tissue receptors is greater at birth than in the mature individual (Table I). There is, however, little information regarding the intrauterine acquisition of enhanced specific binding of insulin and the implications of this phenomenon for the fetus. The present investigation was performed to define the characteristics of hormone–receptor interaction during the last 2 months of gestation in fetal sheep liver and kidney and to relate the observed characteristics to certain postreceptor events.

Materials and Methods. Twenty-one typical western range sheep, primarily a Dorset–Rambouillet mixed breed, were bred for this study. At various gestational ages from 94 days to term at 147 days they were sacrificed by exsanguination. After the fetuses were weighed, fetal livers (n = 46) and kidneys (n = 11) from the 46 fetuses and 11 maternal livers and kidneys were removed immediately and placed in ice-cold isotonic saline. Fetal vertebral column length was measured (11). To prepare a plasma membrane suspension, the organs were dissected, placed in 0.3 M sucrose maintained at 4°C, and processed immediately (12). A Waring blender and a Tekmar tissuemizer with SDT-182 EN shaft and generator were used to homogenize the tissues. Each homogenate was centrifuged at 600g for 10 min at 4°C, and the precipitate was washed with 0.3 M sucrose and recentrifuged. The pooled supernates were centrifuged at 12,000g for 30 min, and the supernate was removed, pooled, and measured for volume. To the supernate were added NaCl and $MgSO_4$ to

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TABLE I. COMPARISON OF ONTOGENIC CHANGES IN INSULIN–PLASMA MEMBRANE RECEPTOR INTERACTION IN VARIOUS TISSUES FROM SEVERAL SPECIES

Species	Tissue	Ontogenic changes: Specific binding	Affinity	Receptor concentration	Reference
Sheep	Erythrocytes	F[a] > N[b] > A[c]	F < N	F > N	(34)
Rabbit	Liver	N > A			(35)
	Liver	F↑[d] ~ Gest[e] F > A	F ≈ N ≈ A	F↑ ~ Gest	(36)
Rat	Liver	F < N < A		F↑ ~ Gest	(37)
	Liver	F ≈ N ≈ A			(35)
	Liver	F↑ ~ Gest F > A	F↑ ~ Gest F > A	F↑ ~ Gest F > A	(38)
Guinea Pig	Liver	F↑ ~ Gest F > A	F > A	F↑ ~ Gest F > A	(28)
	Liver	F ≈ A			(35)
	Kidney	F↑ ~ Gest			(35)
	Myocardium	"Rich" in N			(39)
	Placenta	F↓[f] ~ Gest F↑ ~ wt			(40) (35)
Human	Liver	F↑ ~ Gest	F↑ ~ Gest	F↑ ~ Gest	(38)
	Mononuclear leukocytes	N > A	N > A	N > A	(41)
	Mononuclear leukocytes	F ~ wt	F > A		(42)
	Erythrocytes	F > C[g] > A	F ≈ C ≈ A	F > C > A	(43)
	Erythrocytes	F > C	F > C		(33)
	Erythrocytes	F↓ ~ Gest F > A	F↓ ~ Gest F > A		(44)
	Myocardium	"Rich" in N			(39)
	Placenta	F↑ ~ Gest			(45, 46)
	Brain	F↑ ~ Gest			(47)

[a] F = fetus.
[b] N = neonate.
[c] A = adult.
[d] ↑ = increases.
[e] Gest = gestational age.
[f] ↓ = decreases.
[g] C = child.

achieve final concentrations of 0.1 and 0.001 *M*, respectively. The supernate was again centrifuged at 40,000*g* for 40 min; the pellet was resuspended in 8 vol of 0.025 *M* Tris–HCl buffer, pH 8.0, containing 0.01 *M* Ca, and recentrifuged at 40,000*g* for 40 min. The final pellet was resuspended in 2 vol of cold 0.025 *M* Tris–HCl buffer. An aliquot was set aside for total protein determination, and the remainder was stored in aliquots at −70°C until assayed for insulin binding, usually within 1 week, but never more than 3 weeks after processing. Preliminary studies which compared binding to fresh membrane preparations with that to aliquots that had been frozen and thawed revealed no effect of freezing and thawing when samples were frozen no longer than 3 weeks. These preliminary studies also demonstrated maximal binding at pH 8.0, and buffers of this pH were used subsequently for membrane preparation and binding studies.

The 40,000*g* fraction of homogenized tissues was selected for radioreceptor assays. Preliminary assessment of plasma membrane purity was made by testing 5′-nucleotidase activity (13). The mean value of tissue from four animals sampled at widely different gestational ages showed that the 40,000*g* fraction exhibited 3.4 times the 5′-nucleotidase activity that was exhibited in the original homogenate and

80% of the 5'-nucleotidase activity that was exhibited in the 100,000*g* fraction. The specific binding of insulin to the 40,000*g* fraction (B_{sp}) in the absence of cold insulin was found to be also 80% of binding to the 100,000*g* fraction. The quantity of material in the 100,000*g* fraction for individual sheep fetuses of less than 110–120 days, however, was insufficient for the performance of a complete competitive assay of insulin binding; hence, the partially purified 40,000*g* fraction was used for all samples.

Competitive binding studies using porcine insulin as trace were carried out with ovine insulin (Novo Research Institute, Copenhagen, Denmark); bovine insulin (Schwartz/Mann, Orangeburg, N.Y.); porcine insulin (Lilly Research Laboratories, Indianapolis, Ind.); bovine proinsulin (a gift from Lilly); ovine growth hormone, human growth hormone, ovine prolactin, and human prolactin (all gifts from the National Pituitary Agency, Baltimore, Md.), and porcine glucagon (Cambridge Nuclear Radiopharmaceutical Corp., Billerica, Mass.) (12). These studies demonstrated that the radioreceptor assay for liver and kidney plasma membranes was specific for insulin and that the receptor was much more avidly bound to species specific insulin (ovine > bovine > porcine insulin). Initially, assays of insulin binding to fetal liver plasma membranes were conducted in parallel, using Iletin U-100 porcine insulin (Lilly) and freshly reconstituted ovine insulin. These parallel studies revealed similar changes in insulin binding during the course of gestation; no effect of possible microaggregates nor of phenol in the porcine insulin was detected.

Maximal specific binding of ^{125}I-insulin to membranes in the 40,000*g* fraction was found to occur between 20 and 24 hr of incubation at 4°C. This temperature was chosen because receptor degradation as measured by preincubation was less (20%) at 4°C than at either 25 or 37°C, and because under these conditions mean hormone degradation in four samples in the presence of buffer alone as measured by 10% TCA precipitation and subsequent reincubation of ^{125}I-insulin with fresh membranes was only 1%. Just prior to radioreceptor assay the protein concentration of each tissue fraction was determined by a fluorescamine method (14).

Suspensions of the 40,000*g* fraction containing liver or kidney membranes from each animal at a concentration of 200 μg protein/ml were incubated for 20 hr at 4°C with porcine ^{125}I-insulin at a final concentration of 2×10^{-11} *M* (Cambridge Nuclear, Cambridge, Mass.) and 13 various final concentrations of unlabeled insulin from 0 to 1.72×10^{-6} *M*, (Iletin U-100, porcine, Lilly) in 0.025 *M* Tris–Ca buffer (pH 8.0) and 0.1% bovine serum albumin (RIA grade, Sigma Chemical Company, St. Louis, Mo.). The sp act of the ^{125}I-insulin was 120–160 μCi/μg. The final volume in each tube was 0.5 ml. After incubation the tubes were centrifuged for 10 min at 7000*g* in a Sorvall RC-5 refrigerated centrifuge, the supernatant was discarded, and the pellets were washed twice in cold Tris buffer. The radioactivity of the pellets was counted in a Packard autogamma spectrometer. Percentage nonspecific binding was measured as cpm ^{125}I-insulin in the presence of 1.72×10^{-6} *M* unlabeled insulin divided by the total cpm of ^{125}I-insulin less the cpm bound to the glass × 100. Nonspecific binding was subtracted from all data points, and the data for each organ from each animal were then subjected to Scatchard analysis (15, 16) performed on a Wang 2200T-4 computer (Wang Laboratories, Tewksbury, Mass.). We used the method of DeMeyts and Roth to calculate equilibrium constants for insulin interaction with plasma membranes (15). A linear regression equation for each individual organ was used to calculate the total number of insulin receptors per unit protein, R_0, assuming one molecule of insulin binds to one receptor site. For this system in which site–site interaction has been postulated, we calculated the average affinity constant for empty receptors, $\overline{Ke}$.

An additional 30 sheep fetuses were sacrificed at gestational ages that ranged from 85 to 140 days. Excisional biopsies of fetal liver were immediately obtained and placed on dry ice. These liver samples were stored at −70°C until they were assayed for glycogen content (17).

Statistics. Parametric statistics were used to analyze the results. The unpaired Student *t* test was used to compare differences in means of variables (B_{sp}, $\overline{Ke}$, R_0) between two groups of fetuses of different gestational age. Curve fitting and goodness of fit of linear, exponential, and geometric regression equations to data for B_{sp}, $\overline{Ke}$, and R_0, respectively, as functions

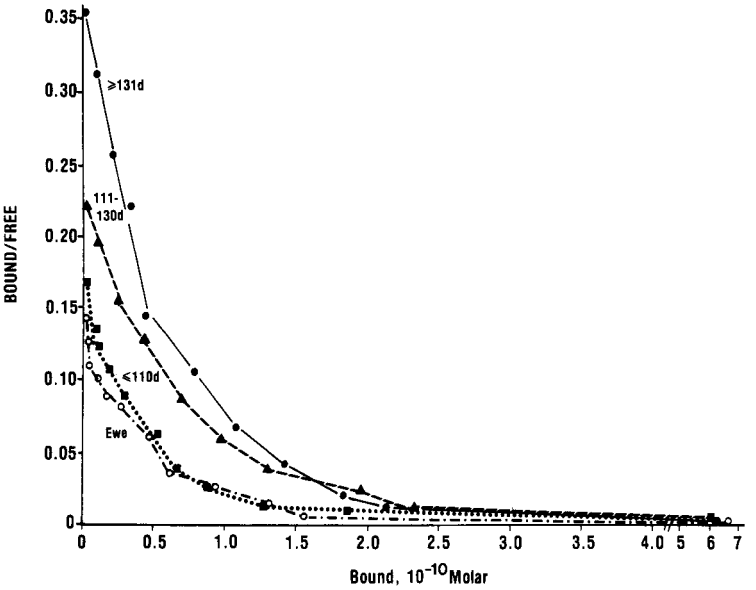

FIG. 1. Scatchard plot of ovine liver plasma membrane insulin receptor interaction with ^{125}I-insulin (2×10^{-11} M) after incubation of membranes (0.1 mg protein/0.5 ml total vol) in the presence of unlabeled insulin (0–1.72×10^{-6} M). Plots for fetal tissues studied at three sequential periods in the last 2 months of gestation and for ewes indicate increasing binding as gestation nears term. (n = 19 for ≤110 days, 13 for 111–130 days, and 14 for ≥131 day fetuses; n = 11 for ewes).

of gestational age were accomplished using the Wang program (18). Multivariate analysis of covariance was performed on a VAX minicomputer (Digital Equipment Corp.) using Statistical Analysis System version 82.4. In the final model used for the analysis of results reported below, vertebral column length and body weight were modeled simultaneously as dependent variables with gestational age and the interaction of fetal sex and specific binding of insulin to hepatic membranes before 120 days and after 120 days gestation. Independence of data from fetuses was assumed, although data from multiple gestation pregnancies are included.

Results. Specific binding of insulin to partially purified ovine fetal liver membranes increased as gestation progressed toward term as illustrated in Fig. 1 and Table II. Throughout the portion of gestation surveyed, specific insulin binding was greater to fetal than to maternal liver membranes. The increased binding to fetal liver membranes that occurred late in gestation (≥131 days) was associated with an increase in the affinity for high-affinity binding sites, $\overline{Ke}$. This association is apparent from the Scatchard plot shown in Fig. 1; Table II reveals that the $\overline{Ke}$ after 131 days was approximately twofold greater than the $\overline{Ke}$ of both fetal liver before 131 days and maternal liver. The insulin receptor concentration in fetal liver membranes did not change during the last 2 months of gestation ($\bar{x} \pm$ SEM R_0 = $183 \pm 26 \times 10^{10}$ sites/mg) and was not different from the R_0 for maternal liver ($148 \pm 41 \times 10^{10}$ sites/mg).

Similarly, specific insulin binding to partially purified fetal kidney membrane was observed to increase as term was approached (Figs. 2 and 3). Although fewer kidneys than

TABLE II. CHARACTERISTICS OF ^{125}I-INSULIN BINDING TO PARTIALLY PURIFIED OVINE LIVER PLASMA MEMBRANES

	Fetal < 131 days	Fetal > 130 days	Ewes
n	32	14	11
Specific binding, %	14.4 ± 1.1[a]	22.1 ± 3.3[b]	10.7 ± 1.5
Ke for high-affinity receptors, 10^8 M^{-1}	6.4 ± 1.1	13.0 ± 2.7[c]	5.2 ± 2

[a] Mean ± SEM.
[b] $P < 0.005$ cf. fetuses < 131 days.
[c] $P < 0.01$ cf. fetuses < 131 days.

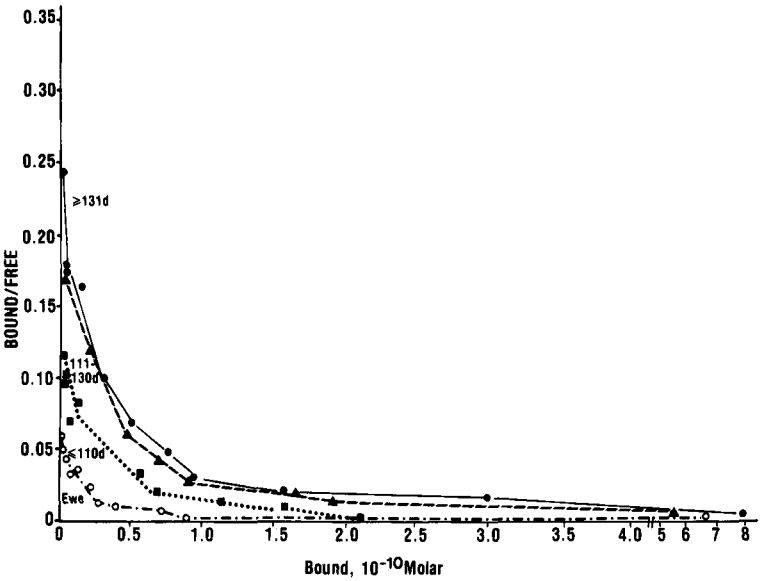

FIG. 2. Scatchard plot of ovine kidney plasma membrane insulin receptor interaction with ^{125}I-insulin (2×10^{-11} M) after incubation of membranes (0.1 mg/0.5 ml total vol) in the presence of unlabeled insulin (0–1.72×10^{-6} M). Plots for fetal tissues studied at three sequential periods in the last 2 months of gestation and for ewes indicate increasing binding as gestation nears term. (n = 4 for ≤110 days, 4 for 111–130 days, and 3 for ≥131 day fetuses; n = 4 for ewes).

livers were studied, the increase in specific binding to fetal kidney membranes appears to occur steadily during the last 45 days of gestation and can be expressed by the linear regression equation shown in Fig. 3 with a correlation coefficient of r = 0.66. Mean insulin specific binding to fetal kidney membrane receptors (14.4 ± 2.0%) was also greater during the portion of gestation investigated than was specific binding to maternal kidney membranes (4.9 ± 0.9%; $P < 0.005$). Analyses of Ke and R_0 for fetal kidney reveal an increase in R_0 between seven fetuses of less than 110 days gestation ($60 \pm 9 \times 10^{10}$ sites/mg) and four fetuses of 111–130 days ($627 \pm 235 \times 10^{10}$ sites/mg; $P < 0.05$) and an increase in Ke from fetuses of 111–130 days ($1.3 \pm 0.5 \times 10^8$ M^{-1}) to three fetuses of more than 130 days ($7.2 \pm 2.2 \times 10^8$ M^{-1}; $P < 0.025$).

Male fetuses (n = 22) were analyzed separately from female fetuses (n = 24) with regard to hormone binding to liver membranes (Fig. 4). The increase in binding of insulin to male fetal liver membranes was exponential; there was no apparent increase in specific binding to female liver membranes over the range of gestation surveyed. Thus, the increase in specific binding of insulin to fetal sheep liver membranes that we observed is attributable solely to the increase in binding by male tissues. There were too few kidney samples to detect differences between male and female tissues (Fig. 3).

In Fig. 5 are shown respectively the fetal vertebral column length (top) and body weight (bottom) as functions of gestational age. These plots indicate that both weight and vertebral column length are functions determined primarily by gestational duration. However, Fig. 5 also suggests that the growth of male sheep fetuses is greater late in gestation than that of female fetuses. We used multivariate analysis to test whether the sex difference in specific binding of insulin to fetal tissues might explain some of the variability in fetal growth after 120 days gestation. The overall model was highly significant ($P < 0.0005$); the individual effects attributed to gestational age and to the specific binding of insulin in females after 120 days gestation were each significant at $P < 0.001$. Gestational age was found to account for 90% of the variance in vertebral column length and 81% of the variance in fetal weight. The interaction between fetal sex and insulin specific binding accounted for 30% of the remaining variance in fetal vertebral column

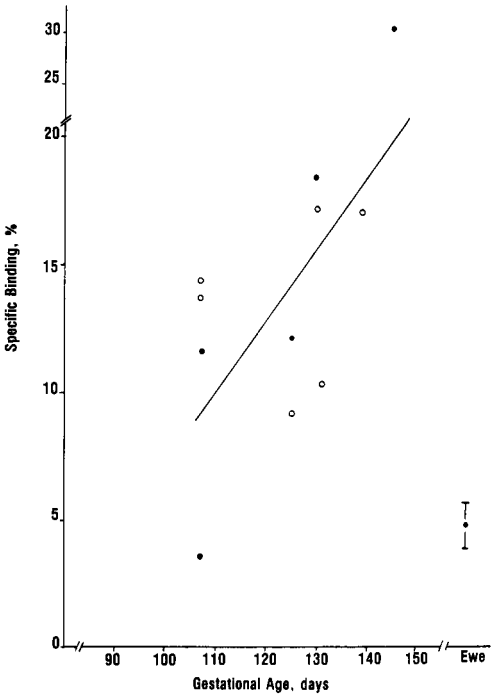

FIG. 3. Percentage specific binding of ^{125}I-insulin, B_{sp}, in the absence of unlabeled insulin to fetal kidney plasma membrane preparations (0.1 mg/0.5 ml total vol) between 107 days and term. Represented by the line is the linear regression equation which describes the increase in B_{sp} with gestational age over this portion of gestation: $y = 0.33x - 25.8$; ($r = 0.66$; $P < 0.05$). For comparison, the mean ± SEM B_{sp} for four maternal sheep kidneys is shown. Data for female fetuses are represented by open circles; data for male fetuses are represented by closed circles.

length and 22% of the remaining variance in fetal weight.

In Fig. 6 is shown the increase in glycogen content in fetal sheep liver that occurred between 85 and 140 days gestation. No sex difference was detected by multivariate analysis.

Discussion. This study demonstrates that insulin binds to its receptors in partially purified sheep fetal liver and kidney plasma membranes prior to 110 days gestation and that specific binding increases late in gestation to two to four times that observed in the respective maternal tissues. The twofold increase in hormone binding to fetal liver is associated with a late gestational increase in affinity, while the progressive fourfold increase in specific binding to kidney is associated with increases initially in receptor concentration and subsequently in affinity. Binding of insulin to male liver membrane receptors increases exponentially as term is approached; female tissues exhibit no change during the gestational interval sampled. Both vertebral column length and fetal weight increase with increasing gestation, but the growth of males is greater than that of females. The interaction between fetal sex and specific binding of insulin to fetal liver membranes can explain 22–30% of the variability in fetal growth that is unexplained by length of gestation.

These observations of the ontogeny of insulin binding to ovine liver and kidney may be compared with the results of similar investigations of insulin binding to other fetal, neonatal, and mature plasma membranes from sheep as well as other species in Table I. Specific binding of insulin to membrane receptors of most tissues is, in general, greater in late fetal specimens than in neonatal tissues, and neonatal tissues in general exhibit greater specific binding than do adult tissues. In many tissues an increase in affinity or receptor concentration, or both, has been associated with the late fetal and neonatal increased specific binding compared with adult values. However, the relationship between developmental changes in insulin hormone-receptor binding to possible postreceptor functional effects has only recently begun to be explored.

In fetal sheep, insulin has been shown to be active during late gestation in regulating blood

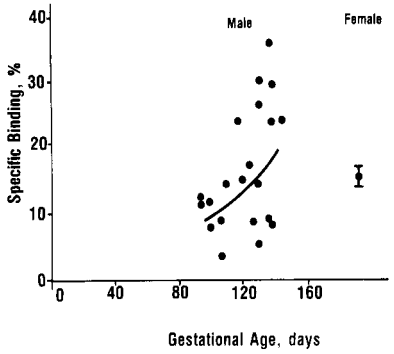

FIG. 4. Percentage specific binding of ^{125}I-insulin to plasma membrane preparations of 22 male fetal sheep livers (0.1 mg/0.5 ml total vol) from midgestation to term. Specific binding to male liver preparations increases logarithmically according to the equation represented by the broken line: $\ln y = 0.016x + 0.68$; ($r = 0.42$; $P < 0.05$). Specific binding to female liver preparations does not change detectably over the last half of gestation; the mean ± SEM value for 24 female fetal sheep livers is shown.

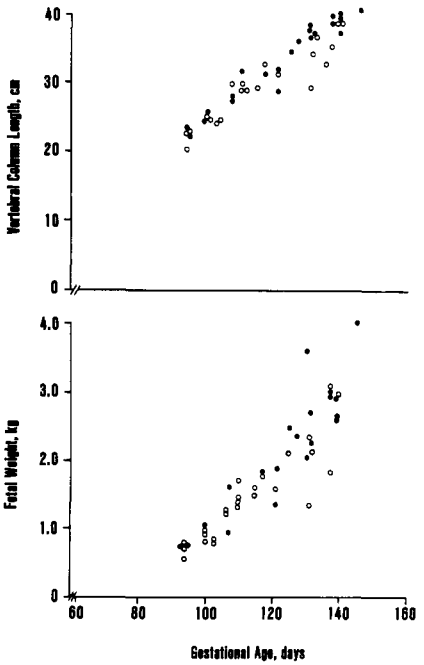

FIG. 5. Fetal sheep vertebral column length (top) and fetal body weight (bottom) for male (closed circles) and female (open circles) fetuses as functions of the length of gestation in sheep. After approximately 120 days male and female fetuses exhibit different growth curves. Multivariate analysis reveals that the interaction of sex and specific binding of insulin to fetal liver membranes after 120 days has significant effects on both fetal vertebral column length and body weight, accounting for 22–30% of the variance of these growth parameters that is not explained by gestational duration.

glucose levels (19, 20), and fetal utilization of glucose (10). However, there is a blunted, slower fetal response in blood glucose concentration to insulin compared with the postnatal and adult responses (19) that suggests diminished hormone effect on muscle and adipose tissues. In mature animals insulin diminishes gluconeogenesis (21), and insulin increases hepatic and placental glycogen synthetase and phosphorylase activities and glycogen synthesis (22–24). Fetal sheep liver and kidney after 120 days gestation have the enzymatic capacity for gluconeogenesis (25), and indeed, gluconeogenesis has been demonstrated in the sheep fetus in late gestation (26), but gluconeogenic activity is less than that in nenonatal lambs and adult sheep. Thus, fetal insulin possibly may inhibit fetal liver and kidney gluconeogenesis in late gestation, and such inhibition may be a manifestation of the increased hormone–receptor interaction which appears to be present prior to birth. Whether the hepatic and renal postreceptor events are fully operational in late gestation and whether fetal insulin does indeed participate in the regulation of fetal gluconeogenesis are aspects of the endocrine control of metabolism which have not yet been studied in this species. There is evidence in the rat that postreceptor maturation occurs during the last 20% of gestation (27) and in the guinea pig that glucagon receptors in fetal liver are uncoupled from the adenylate–cyclase complex (28).

Insulin also promotes hepatic glycogen synthesis in the mature subject. We have considered that the increased binding of insulin to hepatic plasma membranes in late gestation observed in this study may underlie the late-gestation fetal hepatic storage of glycogen (29). We found that insulin binding late in gestation was greater to plasma membranes of liver, the major site of glycogen storage, than to plasma membranes of kidney. Moreover, the increased binding in liver was accomplished by increased affinity, a rapidly reversible aspect of hormone–receptor interaction and one that might permit glycogen to be released from hepatic storage after birth. The exponential increase in insulin binding to hepatic plasma membranes noted in male fetal sheep might be expected to result in greater hepatic glycogen stores in male sheep fetuses compared to female fetuses during very late gestation if postreceptor events were coupled to the receptor. However, we were unable to demon-

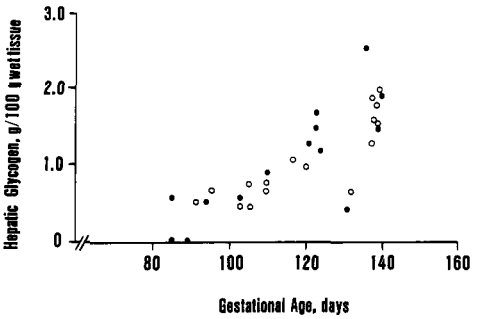

FIG. 6. Hepatic glycogen content in 30 fetal sheep from 85 to 140 days gestation. Male tissues are represented by closed circles; female tissues are represented by open circles. There is no detectable difference between male and female tissues.

strate a difference in glycogen content between male and female tissues. Late gestational fetal rat liver has been found to exhibit evidence of possible dissociation of insulin receptors from the postreceptor synthesis of glycogen (30). Our observations in the sheep suggest that liver insulin receptors may be uncoupled from glycogen synthesis in this species, also, for the increase in liver glycogen content was not different between males and females, although specific binding of insulin was different.

Although the observed sex difference in specific binding of insulin to fetal liver was not manifested in a sex difference in glycogen content, our data suggest that the sex difference in insulin–receptor interaction can explain as much as 30% of the variances in fetal body mass and in fetal skeletal growth which is not explained by gestational duration. This observation does not permit one to conclude, however, that sex differences exist in insulin–receptor interaction with fetal muscle, fat, and/or cartilage cells, or that receptor or postreceptor events are coupled for one or more of these fetal tissues, because the differences in fetal growth might be mediated by hepatic functions other than glycogen synthesis for which the postreceptor events are coupled to the insulin receptor.

The difference between the sexes in hepatic insulin specific binding raises the possibility that some characteristic that distinguishes male fetuses from female fetuses increases the affinity of insulin for hepatic plasma membranes late in gestation in sheep. In this regard, reports of increased binding of insulin to erythrocytes and monocytes from adult human males compared with binding to erythrocytes and monocytes from adult females (31–33) are of interest. However, the increased specific binding observed in the blood cells appears to be related to increased receptor number on male cells rather than to increased affinity, an observation different from that made in the present study of male fetal liver binding characteristics. Additional evidence within females that insulin binding may be affected by sex hormones has been obtained in studies of specific binding to monocytes and erythrocytes of women during phases of the menstrual cycle (31, 32). Specific binding of insulin is greater during the follicular phase than in the luteal phase; again, however, the difference is related to receptor concentration. Insulin binding to monocytes has been shown to be inversely related to levels of 17β-estradiol, progesterone, and 17α-hydroxyprogesterone (32). We are, however, unaware of reported sex differences in these hormones in fetal sheep late in gestation which might account for the differences we observed between male and female tissues.

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