Ontogeny and insulin regulation of fetal ovine white adipose tissue leptin expression

SHERIN U. DEVASKAR,1 RUSS ANTHONY,2 AND WILLIAM HAY, JR.3

Divisions of Neonatology, Departments of Pediatrics, 1University of California Los Angeles School of Medicine, Los Angeles, California 90095; 3University of Colorado, Denver 80217; and 2Department of Physiology, Colorado State University, Fort Collins, Colorado 80523-1683

Received 18 December 2000; accepted in final form 2 October 2001

Devaskar, Sherin U., Russ Anthony, and William Hay, Jr. Ontogeny and insulin regulation of fetal ovine white adipose tissue leptin expression. Am J Physiol Regulatory Integrative Comp Physiol 282: R431–R438, 2002.—Leptin, an adipocyte-derived factor, has multiple biological roles including mitogenesis. We investigated the effect of normal development, acute and chronic hyperglycemia and hypoglycemia, and selective acute hyperglycemia, or hyperinsulinemia, on fetal ovine white adipose tissue (WAT) leptin mRNA concentrations. Leptin mRNA amounts expressed as a ratio to the internal control ribosomal S2 mRNA decreased threefold with advancing gestational age (P < 0.05). This gestational decrease was opposite to the 10-fold increase in fetal body weight during the same developmental period. Chronic hyperglycemia with hyperinsulinemia led to no change in WAT leptin mRNA concentrations over a 1- to 10-day duration, but it caused a 40% increase over a 14- to 20-day duration (P < 0.05) along with an increase in fetal body weight (P < 0.05). In contrast, hypoglycemia with hypoinsulinemia, while not affecting WAT leptin mRNA from 1 to 34 days, resulted in a 50% decline over a 36- to 76-day duration along with a decline in fetal body weight (P < 0.05). Acute 24-h studies of selective hyperglycemia with euglycemia showed no significant change in WAT leptin mRNA, but in response to selective hyperinsulinemia with euglycemia at 24 h, a twofold increase was observed (P < 0.05). We conclude that fetal WAT leptin mRNA amounts are regulated by fetal development and circulating insulin concentrations. We speculate that chronic in utero metabolic perturbations that alter circulating insulin concentrations affect fetal leptin production that may mediate insulin’s influence on fetal growth.

GLUCOSE; OBESITY

LEPTIN IS A 16-kDa circulating peptide that is predominantly synthesized by adipocytes (6, 39). Upon interaction with various alternately spliced isoforms of the receptor (16, 35), leptin mediates a multitude of biological actions in the adult (11, 23). One of its biological effects includes regulation of body weight by controlling hypothalamic mechanisms involved in food intake (4) and sympathetic mechanisms that regulate energy expenditure (32, 36). Leptin has also been noted to inhibit insulin secretion by the pancreatic β-cell (26, 40) and to increase peripheral insulin sensitivity (24, 37). While investigations during development are limited, there is emerging evidence to support roles for leptin in the fetal and immediate postnatal period (19, 38), consisting of growth potentiation in the fetus (19), body weight loss (38), and disinhibition of nonshivering thermogenesis in the neonate (5). In the human, however, umbilical cord leptin concentrations correlate with birth weight, being high when an infant is macrosomic and low with intrauterine growth restriction (25, 34). These reports suggest that fetal leptin concentrations are associated with the intrauterine growth potential. Observations in an infant of a diabetic mother revealed that circulating leptin concentrations are higher than that observed in a macrosomic infant born to a nondiabetic mother (15, 27). These observations indicate that the fetal metabolic and hormonal milieu independent of the fetal fat content may regulate leptin synthesis and circulating concentrations. On the basis of this information, we hypothesized that advancing gestational age with an increase in fetal body weight will be associated with increasing fetal adipose tissue leptin expression. Furthermore, fetal hyperglycemia and/or hyperinsulinemia would enhance adipose tissue leptin expression, whereas hypoglycemia with hypoinsulinemia would diminish the same. To test these hypotheses, we initially determined fetal white adipose tissue (WAT) leptin expression at different gestational ages, thereby delineating the ontogeny. We next carried out chronic fetal hyperglycemia and hypoglycemia studies using maternal glucose and insulin infusions, respectively, in catheterized pregnant sheep. On the basis of the results of the chronic fetal hyperglycemia studies, we subsequently undertook acute fetal studies involving selective hyperglycemia or hyperinsulinemia using glucose or insulin infusions, respectively.

MATERIALS AND METHODS

Animals

Columbia-Rambouillet mixed-breed pregnant ewes, each carrying a single fetus, were obtained from Nebeker Ranch (Santa Monica, CA). Sheep were kept in separate carts, but at least two sheep were kept in the same room for company. All studies, animal surgery, and animal care procedures were

Address for reprint requests and other correspondence: S. U. Devaskar, Dept. of Pediatrics, Univ. of California Los Angeles School of Medicine, 10833 LeConte Ave., Los Angeles, CA 90095.

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in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the University of Colorado Health Sciences Center Institutional Animal Care and Use Committee. The animals were cared for and were studied in the University of Colorado Health Sciences Center Perinatal Research Facility, which is accredited by the NIH, the United States Department of Agriculture, and the Association for the Assessment and Accreditation of Laboratory Animal Care.

Surgery. Surgery was done at ~115–120 days of gestation (term 150 days) to place maternal and fetal infusion and blood-sampling catheters according to standard procedures (12). After all wounds were closed, the catheters were tunneled subcutaneously through a skin incision and kept in a plastic pouch attached to the ewe’s flank. Each ewe received intramuscular gentamicin (80 mg) and penicillin G procaine (600,000 U), and the fetus was treated with intra-amniotic ampicillin (500 mg) at the time of surgery. Postoperatively, each ewe was maintained in its own cart and allowed ad libitum access to water, alfalfa pellets, and a mineral block. The ewes were maintained under environmental conditions of 20 ± 1°C with 18 h of light and 6 h of darkness daily. Weekly intramuscular injections of a multivitamin preparation were given (B-complex vitamins; Vedco, St. Joseph, MO). The catheters were flushed daily with 1.5 (fetal) or 3.0 (maternal) ml of heparinized saline solution (150 U heparin/ml in 0.9% wt/vol NaCl in H2O). Each animal was allowed at least 4 days to recover from surgery before chronic or acute infusions were begun (12).

Chronic Studies

Hyperglycemia study. The pregnant ewes were made chronically and markedly hyperglycemic (n = 25) by a continuous 50% dextrose (wt/vol in H2O) intravenous infusion at a variable rate that was adjusted to maintain maternal arterial blood glucose concentration at relatively high and constant levels (Δglucose above euglycemia = 2 mM or twice normal values) in response to measurements of blood glucose concentration made twice daily (model 2700 glucose analyzer, Yellow Springs Instrument (YSI), Yellow Springs, OH) (12). This target glucose concentration was chosen to maintain a physiological rather than a pharmacological perturbation. Differing durations of hyperglycemia ranging from 1-2 to 16–20 days were used to test for adaptation to chronic conditions. Longer studies could not be done for the hyperglycemia group due to more complications with catheter loss secondary to increased thrombosis (12).

Hypoglycemia study. Insulin was infused into the ewes to produce acute and/or chronic hypoglycemia (n = 14) (12). Insulin (regular insulin, Iletin U-100; Eli Lilly, Indianapolis, IN) was diluted in normal saline [0.9% (wt/vol) NaCl in H2O] and administered by constant infusion (~20 ml/day) at 30–60 nmol (5–10 mU)·min–1·kg maternal wt–1 in 0.9% (wt/vol) NaCl in H2O as previously described (12). The insulin infusion rate was adjusted daily in response to measurements of maternal glucose concentrations to produce hypoglycemia values ~50% of normal (1.4–1.5 mM). This target glucose concentration was chosen to maintain a physiological rather than a pharmacological perturbation. Differing durations of hypoglycemia ranging from 1–2 to 36–76 days were undertaken to test for adaptation to chronic conditions.

Euglycemic controls. Euglycemic (~2.8 mM) and normoinsulinemic, gestationally age-matched, and catheterized (maternal and fetal) pregnant ewes (n = 31) constituted the sham control group. These animals were operated on, monitored, and treated exactly as the experimental animals over the same durations as the hyperglycemia and hypoglycemia animals except for the continuous intravenous infusions.

Acute Studies

Animals were equally divided into two study groups. One group of fetuses was made hyperglycemic while maintaining normal maternal insulin concentrations, and the other was made hyperinsulinemic while maintaining normal plasma glucose concentrations (1). These two study groups were each subdivided into three experimental groups according to the duration of hyperglycemia or hyperinsulinemia over 1 h (to study an immediate effect), 2.5 h (to examine a time frame when the earliest mRNA changes may be observed), or 24 h (to detect mRNA changes that manifest later). Control blood samples for fetal and maternal glucose and insulin concentrations were obtained four times at 10-min intervals. Basal glucose concentration, or fetal euglycemia, was defined as the mean fetal arterial plasma glucose concentration measured in the four control samples.

Hyperglycemia studies. Fetal hyperglycemia with normal fetal plasma insulin concentration was produced by infusing 50% dextrose in water (D50W) into the mother and somatostatin into the fetus (n = 13) to prevent increased fetal insulin secretion in response to experimental hyperglycemia. Somatostatin (6 mg) was mixed in 0.9% wt/vol sodium chloride in water (20 ml) for a somatostatin concentration of 300 μg/ml. Somatostatin was given as a bolus to the fetus (100 μg/kg) followed by constant infusion (4 μg·min–1·kg–1). Maternal glucose clamp technique was used to keep fetal arterial plasma glucose concentration at twice the mean control period value (~40 mg/dl (2.22 mM) for the study). The dextrose infusion into the ewe was started 60 min after starting the somatostatin infusion into the fetus. The ewe received a priming bolus infusion of D50W [~330 mg (18.3 mM) dextrose/kg maternal weight] followed by a variable infusion of D50W, beginning with an infusion rate of 20 ml/h (~3.7 mg (0.21 mM) dextrose·min–1·kg maternal weight–1). Fetal arterial plasma glucose concentration was measured every 10 min, and the maternal glucose infusion rate was adjusted until fetal plasma glucose concentration was stable at the target concentration. Hyperglycemic clamp was maintained for the duration of the study by small changes in the rate of glucose infusion into the ewe made intermittently in response to measured fetal arterial plasma glucose concentrations (1).

Hyperinsulinemia studies. Fetal hyperinsulinemia with euglycemia was produced by infusing insulin into the fetus (n = 12) and adjusting the dextrose infusion into the ewe to keep fetal plasma glucose concentration at baseline. Regular insulin (100 U/ml) was mixed with normal saline to a concentration of 60 μU/ml. The fetus received a 30 μU/kg insulin bolus followed by infusion at 1 μU·min–1·kg–1. Fetal arterial plasma glucose concentration was measured every 10 min, and the dextrose infusion rate into the ewe was adjusted until the fetal arterial plasma glucose concentration was not different from baseline and remained relatively stable. Fetal euglycemia was maintained for the duration of the study by small changes in the maternal dextrose infusion rate made intermittently in response to measured fetal arterial plasma glucose concentrations (1).

In both study conditions and before starting any infusions, 15 ml of maternal blood were drawn into a syringe containing 1 ml of heparin (1,000 U/ml). This was used to replace fetal blood loss from phlebotomy. Transfusion to the fetus occurred over 10 min immediately following the control blood sampling.
After clamp infusion periods of 1, 2.5, or 24 h, experimental fetal and maternal samples were obtained at four times, 10 min apart. Assays included fetal and maternal arterial plasma glucose and insulin concentrations. Fetal arterial oxygen saturation was measured at the beginning and at the end of the control and experimental blood-drawing periods to verify fetal wellness. Study infusions were maintained to the end of the control and experimental blood-drawing periods to measure plasma glucose and insulin concentrations. Fetal arterial samples were obtained at four times, 10 min apart. Assays included fetal and maternal arterial plasma glucose and insulin concentrations. Assays were performed using ovine insulin standards (Eli Lilly). Blood oxygen saturation was measured in duplicate using a YSI model 2700 analyzer. To measure plasma insulin concentrations, blood samples were immediately centrifuged at 1,000 × g until 4°C were obtained within 5 min of induction of anesthesia. After the tissue was obtained, the ewe and fetus were injected with lethal doses of euthanasia (Sleepaway pentobarbital sodium, Fort Dodge Laboratories, Fort Dodge, IA). Samples were immediately snap-frozen in liquid nitrogen and stored at −70°C until analysis.

Biochemical Assays

Plasma glucose concentrations were measured in duplicate using a YSI model 2700 analyzer. To measure plasma insulin concentrations, blood samples were immediately centrifuged at 4°C for 3 min, and the plasma was stored at −70°C until analysis with a Linco rat insulin RIA kit (St. Charles, MO) using ovine insulin standards (Eli Lilly). Blood oxygen saturation and hemoglobin concentration were measured using a Radiometer OSM3 hemoximeter (Copenhagen, Denmark) (12).

**WAT Leptin mRNA Quantification**

Total RNA was isolated from the fetal ovine perirenal WAT according to the method of Chomczynski and Sacchi (10). RNA was quantitated spectrophotometrically, and the purity of the samples was assessed as a ratio of 1.8–2.0 at a 260- to 280-nM wavelength. Total RNA (10 μg) was loaded on 1.2% agarose-2.2 M formaldehyde slab gels and electrophoresed overnight at 22 V for 16 h. The gels were stained with 0.1% ethidium bromide and visualized under ultraviolet (UV) light to confirm the integrity of the RNA samples. The RNA from the gels was transferred to GenScreen Plus membranes and cross-linked by UV light in a Stratagene (La Jolla, CA). To generate the cDNA probes, 1 μg of tRNA from adult sheep WAT was used with oligo(dT) primers in a reverse-transcription (RT) reaction, and the PCR reaction was undertaken at 56°C for annealing, 72°C for extension, and 94°C for melting temperatures with 35 cycles of amplification using primers that were designed from the bovine leptin gene sequence (GenBank accession no. U50365) (primers: sense, bases 1122–1139 = 5’-CCT GTA TCG ATT CCT GTG-3’; antisense, bases 3387–3404 = 5’-TTT CTT CCC TGG ACT TTG-3’). A 528-bp fragment of the ovine leptin cDNA fragment was inserted into a pCR2.1 vector (Invitrogen) and sequenced in both directions to verify authenticity. The sequence matched the partial ovine leptin cDNA (GenBank accession no. U84247). The leptin cDNA was released from the vector by EcoRI enzyme digestion, gel purified, and labeled by random priming (Boehringer Mannheim), and used as a probe.

The blots containing the RNA were prehybridized for 2 h at 42°C and subsequently hybridized overnight at 42°C in 50% formamide, 0.65 M NaCl, 50 mM Tris at pH 7.5, 2 mM Na pyrophosphate, 0.2% polyvinyl pyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, and 1% SDS containing 1.68 × 106 cpm/ml of the leptin cDNA. The blots were washed two times at room temperature in 2× SSC/0.5% SDS and then washed three times for 10 min each at 65°C. The signals on blots were detected in a Molecular Analyst PhosphorImager. The blots were stripped twice at 90°C × 15 min each in 5 mM Tris-HCl at pH 7.0, 0.2 mM EDTA, 0.1% SDS, and reprobed with an S2 ribosomal probe as an internal control (33) to correct for interlane loading variability. The mRNA band density was assessed by densitometry once the presence of linearity between the time of autoradiographic exposure and the optical density was established. The results were expressed as a ratio between the leptin mRNA and S2 mRNA band densities (7).

**Data Analysis**

All data are expressed as means ± SE. When two groups were compared, the Student’s *t*-test was used. When more than two groups were compared, one-way ANOVA followed by the post hoc Newman-Keuls test was employed to detect intergroup differences. In addition, nonparametric testing using the Kruskal-Wallis test followed by a post hoc *t*-test was performed. Significance was achieved at *P* < 0.05.

## RESULTS

**Developmental Study**

Leptin mRNA (4.5 kb) concentrations expressed as a ratio to the ribosomal S2 mRNA to correct for interlane loading discrepancies demonstrated gestational age-related differences. Leptin mRNA levels peaked at gestational age 75–110 days when the body weight was the lowest. Fetal body weight increased with gestational age (Table 1), whereas white fat leptin mRNA concentrations declined from 75–110 to 145–155 days (*P* < 0.05) (Fig. 1). Fetal glucose and insulin concent-

### Table 1. Developmental studies

<table>
<thead>
<tr>
<th>Gestational Age, days</th>
<th>n</th>
<th>Body Weight, g</th>
<th>Plasma Glucose, mg/dl</th>
<th>Plasma Insulin, μU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>78–107 (96.3 ± 9.2)</td>
<td>3</td>
<td>785 ± 284</td>
<td>24.6 ± 0</td>
<td>not collected</td>
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<tr>
<td>129–135 (133 ± 0.9)</td>
<td>6</td>
<td>2,811 ± 137e</td>
<td>19.1 ± 0.89</td>
<td>12.70 ± 2.52</td>
</tr>
<tr>
<td>136–139 (138 ± 0.34)</td>
<td>11</td>
<td>3,373 ± 218e</td>
<td>17.72 ± 1.58</td>
<td>9.64 ± 2.19</td>
</tr>
<tr>
<td>140–144 (141 ± 0.52)</td>
<td>8</td>
<td>3,578 ± 270e</td>
<td>18.94 ± 1.83</td>
<td>13.21 ± 4.14</td>
</tr>
<tr>
<td>145–155 (151 ± 2.08)</td>
<td>3</td>
<td>5,197 ± 46e</td>
<td>20.52 ± 2.35</td>
<td>29.70 ± 4.0*</td>
</tr>
<tr>
<td>P (ANOVA)</td>
<td>0.001</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P* < 0.05 vs. 78- to 107-day values except for insulin vs. the 129- to 135-day values. NS, not significant.
Chronic hyperglycemia and hypoglycemia studies

Table 2. Chronic hyperglycemia and hypoglycemia studies

<table>
<thead>
<tr>
<th>Age, days</th>
<th>Duration, days</th>
<th>Body Weight, g</th>
<th>Plasma Glucose, mg/dl</th>
<th>Plasma Insulin, μU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>135 ± 1.2 (3)</td>
<td>1.38 ± 0.62 (1–2 range)</td>
<td>3,655 ± 493</td>
<td>37.05 ± 0.85‡</td>
<td>13.85 ± 0</td>
</tr>
<tr>
<td>137 ± 0.68 (5)</td>
<td>8.2 ± 0.8 (6–10 range)</td>
<td>3,461 ± 134</td>
<td>43.15 ± 3.44‡</td>
<td>21.01 ± 3.77‡</td>
</tr>
<tr>
<td>137 ± 0.56 (9)</td>
<td>14.3 ± 0.17 (14–15 range)</td>
<td>3,654 ± 300</td>
<td>40.30 ± 1.86‡</td>
<td>16.6 ± 3.36‡</td>
</tr>
<tr>
<td>138 ± 0.78 (8)</td>
<td>17.9 ± 0.55 (16–20 range)</td>
<td>3,897 ± 83‡</td>
<td>44.7 ± 21.33*</td>
<td>22.63 ± 3.35‡</td>
</tr>
<tr>
<td>132 ± 3.5 (2)</td>
<td>1.5 ± 0.5 (1–2 range)</td>
<td>2,781 ± 310</td>
<td>11.85 ± 0‡</td>
<td>not collected</td>
</tr>
<tr>
<td>136 ± 0.67 (3)</td>
<td>12.7 ± 1.3 (10–14 range)</td>
<td>2,969 ± 115</td>
<td>13.42 ± 3.7</td>
<td>8.16 ± 1.04</td>
</tr>
<tr>
<td>133 ± 4.7 (3)</td>
<td>30.7 ± 2.8 (25–34 range)</td>
<td>1,825 ± 44‡</td>
<td>11.21 ± 0‡</td>
<td>not collected</td>
</tr>
<tr>
<td>137 ± 0.79 (4)</td>
<td>44 ± 6.4 (36–76 range)</td>
<td>2,244 ± 148‡</td>
<td>12.07 ± 1.44‡</td>
<td>5.15 ± 1.37*</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>3,175 ± 161</td>
<td>18.20 ± 1.06</td>
<td>11 ± 1.64</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, †P < 0.01, ‡P < 0.001 vs. controls.

Chronic Studies

Hyperglycemia. Maternal glucose infusions produced maternal and fetal hyperglycemia throughout the study period. Mild hyperinsulinemia was noted from 6 to 20 days following the initiation of glucose infusions (Table 2), as opposed to moderate or severe hyperinsulinemia that ensues in response to insulin infusions (17). The fetal body weights were greater than gestational age-matched controls (Table 2) at the time of tissue sampling, achieving statistical significance between 16 and 20 days following initiation of the glucose infusion. Compared with gestational age-matched controls, the perirenal white fat leptin mRNA/S2 mRNA concentration demonstrated no significant change up to 6–10 days (8.2 ± 0.8 days) of fetal hyperglycemia. Between 14 and 20 days of fetal hyperglycemia (mean of 14.3 ± 0.17 and 17.86 ± 0.55 days), however, perirenal fat leptin mRNA concentration increased by 30% compared with gestational age-matched controls (P < 0.05) (Fig. 2A).

Hypoglycemia. Maternal insulin infusions produced maternal and fetal hypoglycemia beginning on the first day of the infusion. Secondary fetal hypoinsulinemia was noted between 36 and 76 days of the hypoglycemic period (Table 2). Fetal body weights were significantly lower than the age-matched controls when autopsied after 25–76 days of the study period (P < 0.05) (Table 2). When the fetal white fat leptin mRNA concentrations were compared with their respective gestational age-matched controls, no difference was noted at 1–2 days (mean of 1.5 ± 0.5 days), 10–14 days (12.7 ± 1.3 days), and 25–34 days (30.7 ± 2.8 days). At 36–76 days (44 ± 6.41 days) of hypoglycemia, there was a 50% decline in fetal leptin mRNA (P < 0.05) compared with gestational age-matched controls (Fig. 2B).

Acute Clamp Studies

Hyperglycemia with an insulin clamp. Hyperglycemic, normoinsulinemic clamp studies revealed no effect on fetal WAT leptin mRNA (Fig. 3). Fetal glucose concentrations were higher than age-matched controls, whereas fetal insulin concentrations were not different from basal values. Fetal body weights were not different from age-matched controls (Table 3).

Hyperinsulinemia with a glucose clamp. Hyperinsulinemic, euglycemic clamp studies revealed that fetal WAT leptin mRNA concentrations increased at 24 h compared with gestational age-matched basal values (P < 0.05) (Fig. 3). Fetal glucose concentrations were not different from basal values, whereas insulin concentrations were significantly higher at all three time points (Table 3). Fetal body weights at the time of tissue collection were not different from age-matched controls (Table 3).

DISCUSSION

We delineated for the first time the ontogeny of fetal ovine WAT leptin expression. An age-dependent de-
cline in fetal WAT leptin mRNA concentrations was observed over the second half of gestation. This decline in leptin mRNA was opposite the age-related increase in fetal body weight. Fetal human adipogenesis, the differentiation of preadipocytes into cells capable of synthesizing and storing triglycerides as fat, usually occurs during midgestation (28), whereas fat deposition occurs in late gestation. Although no information regarding ovine adipogenesis exists, peak fetal ovine WAT leptin mRNA concentrations coincided with the timing of fetal adipogenesis rather than deposition as determined in other species (28). Studies in the human fetus/newborn demonstrate that there is no change in circulating leptin concentrations between 14–32 and 38 wk (3.8 ± 0.3 μg/l), although a relative increase occurs after 38 wk (5.6 ± 0.7 μg/l) (14). However, circulating leptin concentrations may not reflect the fetal WAT production of leptin alone. Although maternal leptin does not cross the placenta (16 kDa) (2, 18), leptin mRNA and protein have been described in the human syncytiotrophoblast and villous vascular endothelial cells (2, 22). This localization suggests that placental leptin can enter the fetal and maternal circulations, thereby contributing to the net fetal and maternal circulating leptin concentrations. Previous

![Fig. 2](image)

**Fig. 2.** A: fetal WAT leptin/S2 mRNA ribosomal ratio at different durations (mean values) of maternal glucose infusion that caused fetal hyperglycemia with hyperinsulinemia. *P < 0.05 compared with the age-matched control group. Inset: representative Northern blots showing leptin (L) mRNA (top) and S2 ribosomal mRNA (S; bottom) in control and hyperglycemic groups. B: fetal WAT leptin/S2 ribosomal mRNA ratio at different durations (mean values) of maternal insulin infusion that caused fetal hypoglycemia with hypoinsulinemia. *P < 0.05 compared with the age-matched control group. Inset: Northern blots showing L mRNA (top) and S mRNA (bottom) in control and hypoglycemic groups.

![Fig. 3](image)

**Fig. 3.** Fetal WAT leptin/S2 mRNA ribosomal ratio at different times of hyperglycemic, normoinsulinemic clamp (HG) and hyperinsulinemic, euglycemic clamp (HI). *P < 0.05 compared with the 0-h value for a given group. Inset: representative Northern blots showing L and S mRNA in the HG and HI groups at different times. Although intragroup and intratime point variability exists between samples as seen in these representative Northern blots, the bar graph represents the mean value of all the samples examined per group and per time point.

### Table 3. Selective hyperglycemia and hyperinsulinemia studies

<table>
<thead>
<tr>
<th>Gestational Age, days</th>
<th>Body Weight, g</th>
<th>Plasma Glucose, mg/dl</th>
<th>Plasma Insulin, μU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hyperglycemia with euinsulinemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-h study</td>
<td>133 ± 1.08 (6)</td>
<td>3,232 ± 255</td>
<td>40.94 ± 4.16‡</td>
</tr>
<tr>
<td>2.5-h study</td>
<td>133 ± 0.8 (3)</td>
<td>3,480 ± 518</td>
<td>33.8 ± 2.43‡</td>
</tr>
<tr>
<td>24-h study</td>
<td>135 ± 1.49 (4)</td>
<td>3,206 ± 239</td>
<td>40.43 ± 2.18†</td>
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<tr>
<td><strong>Hyperinsulinemia with euglycemia</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1-h study</td>
<td>135 ± 1.76 (3)</td>
<td>3,483 ± 268</td>
<td>19.04 ± 5.4</td>
</tr>
<tr>
<td>2.5-h study</td>
<td>132 ± 2.02 (4)</td>
<td>3,565 ± 354</td>
<td>24.25 ± 2.45</td>
</tr>
<tr>
<td>24-h study</td>
<td>134 ± 0.73 (5)</td>
<td>2,927 ± 158</td>
<td>19.26 ± 0.70</td>
</tr>
<tr>
<td><strong>Basal values</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0-h study</td>
<td>136 ± 0.16 (17)</td>
<td>3,175 ± 161</td>
<td>18.20 ± 1.06</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers in parentheses are the number of subjects. *P < 0.05, †P < 0.01, ‡P < 0.001 vs. the basal values.
studies in pig fetuses revealed the presence of adipose tissue leptin mRNA, which peaked at 105 days gestation (9). In contrast to humans and pigs (9, 14), the ovine fetus revealed an earlier peak in WAT leptin mRNA concentrations. This ontogenic change did not parallel any age-related change in fetal glucose or insulin concentrations. Thus the timing of leptin synthesis by fetal WAT or the factors that regulate its synthesis remain unknown.

Previous postnatal human ontogenic studies support the placenta as a major contributor to umbilical venous-circulating leptin concentrations, because cord blood venous levels were considerably higher than those at day 3 of life (2, 20, 22). Furthermore, cord levels of leptin correlated with the size of the infant; namely the levels were high in the macrosomic, large for gestational age infants, and low in the growth retarded, small for gestational age infants (25, 34). Of note, the leptin levels in infants born to diabetic mothers were far greater than those in infants large for gestational age and born to nondiabetic mothers (15, 27). These observations correlated with the findings of the highest leptin mRNA and protein concentrations in the placenta of insulin-dependent diabetic mothers compared with normal infants and of the lowest leptin mRNA and protein concentrations in the placenta of growth-restricted fetuses (22). However, there is no information regarding changes in fetal WAT leptin mRNA concentrations in such conditions.

Our present study examined the chronic effects of hyperglycemia and hyperinsulinemia and observed a time-dependent increase over an ~20-day period in fetal WAT leptin mRNA levels. In contrast, hyperglycemia with hypoinsulinemia revealed a time-dependent (~44 days) decline in fetal WAT leptin mRNA concentrations. This time dependency of the changes in leptin mRNA levels appeared to coincide with the changes in fetal insulin concentrations rather than the glucose levels. Thus, in our chronic hyperglycemia experiments, although fetal hyperglycemia set in by 1–2 days, the increase in fetal WAT leptin mRNA concentrations only occurred at ~14 days, coinciding with the timing of fetal hyperinsulinemia. Similarly, although fetal hypoglycemia set in by 1–2 days, a decline in fetal WAT leptin mRNA concentrations was only evident when hypoinsulinemia was observed at approximately day 44. Our attempt at determining the acute effects of glucose and insulin separately on fetal WAT leptin mRNA confirmed this regulatory role for insulin as opposed to glucose. In the adult, insulin stimulates WAT leptin synthesis (3), whereas leptin, in turn, suppresses β-islet production of insulin (26, 40) and improves insulin sensitivity (24, 37). In the fetus, we observed that insulin plays a similar role in stimulating WAT leptin synthesis. However, unlike the human placenta, we have been unable to detect leptin mRNA in the late-gestation ovine placenta even by RT-PCR (negative data not shown). Thus fetal WAT may prove to be the only major source of circulating leptin in late-gestation sheep.

Combining the results from previous studies in other species (2, 22) and our present study, it appears that in conditions associated with fetal hyperinsulinemia, both placenta in the case of the human (22) and WAT (in the case of sheep and humans) express higher levels of leptin. In contrast, conditions associated with fetal hypoinsulinemia result in a decrease in human placental (22) and fetal ovine WAT leptin mRNA concentrations. Thus, both the placenta and fetal WAT might contribute to circulating leptin concentrations in the human fetus, whereas in late-gestation sheep fetus, WAT may prove to be the predominant if not the only major source.

The biological role(s) of leptin in the fetus has not been thoroughly investigated. During the postnatal stage of development, absence of leptin action in the fa/fa rat leads to obesity (21), whereas exogenous administration of leptin causes a decline in body weight (38). This decline in body weight is partly related to disinhibition of nonshivering thermogenesis and an increase in energy expenditure (5, 38). In the adult, leptin suppresses food intake that contributes to a loss in net body weight (4, 36). Although similar effects have not been observed in the newborn animal, pathways that mediate leptin’s effect on hypothalamic control of appetite are in place during this developmental phase (29, 31). An example of one such mechanism is leptin’s suppression of neuropeptide Y mRNA and peptide, an orexigenic neuropeptide that leads to hyperphagia and obesity in the adult (4). In the fetus, there is a lack of significance to nutritive ingestion, because the nutrient supply is mainly derived from the maternoplacental circulation. However, recent studies in fetal sheep demonstrated that neuropeptide Y increases fetal sheep ingestive behavior, resulting in increased urea output (30). One might speculate that fetal swallowing of amniotic fluid, indicative of ingestive behavior, could be inhibited by the suppressive effect of fetal leptin concentrations on hypothalamic neuropeptide Y concentrations, thereby influencing the net amniotic fluid volume and the developmental state of the gastrointestinal tract.

Changes in fetal WAT leptin mRNA levels may translate into alterations in circulating leptin concentrations. Circumstances that enhance fetal WAT leptin mRNA may alter fetal leptin physiology, which, in turn, may affect the immediate postnatal feeding behavior, energy expenditure, and insulin sensitivity. Thus hyperinsulinemic states associated with high circulating leptin concentrations can suppress milk intake, increase energy expenditure, and increase insulin sensitivity. In contrast, in utero hypoinsulinemic states with low leptin concentrations might lead to postnatal hyperphagia, decreased energy expenditure, and decreased insulin sensitivity. In separate rat studies, we observed that fetal hyperglycemia and hyperinsulinism suppress hypothalamic neuropeptide Y concentrations (31), whereas intrauterine growth restriction with hypoglycemia and hypoinsulinemia leads to persistently increased hypothalamic neuropeptide Y mRNA and peptide concentrations (29).
These observations are consistent with the presence of normal leptin receptor activity rather than leptin resistance, which has been reported in the adult with hyperleptinemia and leptin hyporesponsiveness in the case of hypoleptinemia. Thus, unlike the adult (11, 13, 23), regardless of the in utero changes in leptin concentrations, the lack of chronicity in these in utero leptin perturbations (as they only occur toward late gestation) may not impair the fetal/neonatal leptin receptor sensitivity. Similarly, in separate fetal sheep experiments, similar in utero conditions were associated with an increase in glucose utilization in the case of hyperinsulinemia (17) and a decrease in fetal glucose utilization in the case of hypoinsulinemia (8). Thus it is feasible that fetal leptin concentrations may modulate the state of fetal insulin sensitivity with respect to glucose utilization, which, in turn, could affect fetal growth. In addition, similar to insulin, leptin has been reported to be mitogenic in several cell types (2). Thus fetal hyperinsulinemia-induced hyperleptinemia may be responsible for increased mitogenesis, resulting in an increase in fetal body weight, whereas hypoinsulinemia causing hypoleptinemia may decrease fetal body weight, as we observed in the current study.

In summary, we delineated the ontogeny of fetal sheep WAT leptin mRNA and demonstrated peak levels between 75 and 110 days gestation. In addition, we demonstrated time-dependent effects of hyperglycemia with hyperinsulinemia and hypoglycemia with hypoinsulinemia on fetal WAT leptin mRNA levels. Furthermore, we noted that changes in circulating insulin rather than glucose are responsible for regulating fetal WAT leptin mRNA concentrations.

**Perspectives**

Fetal WAT synthesizes leptin, and in late-gestation sheep, this forms a predominant source of circulating fetal leptin. Fetal WAT leptin mRNA is regulated by the process of development and by perturbations in the metabolic and/or the hormonal milieu. Our studies demonstrated that circulating insulin concentrations are directly proportional to WAT leptin mRNA concentrations. These observations suggest that fetal insulin can stimulate WAT leptin synthesis. Future studies must focus on the biological role of leptin in regulating fetal ingestive behavior, thermogenesis, energy expenditure, insulin secretion, insulin sensitivity, and fetal growth. In addition, the role of fetal leptin in predetermining the fetal-neonatal transitional state that involves establishment of feeding behavior and thermogenesis remains to be explored.

We acknowledge the technical assistance of T. Nguyen.

This work was supported by National Institutes of Health Grants HD-25024 and HD-33997 (to S. U. Devaskar) and HD-20761 and DK-52138 (to W. Hay, Jr.).

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