Maternal obesity and overnutrition alter fetal growth rate and cotyledonal vascularity and angiogenic factor expression in the ewe

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Maternal obesity and overnutrition alter fetal growth rate and cotyledonal vascularity and angiogenic factor expression in the ewe. Am J Physiol Regul Integr Comp Physiol 299: R249–R258, 2010. First published April 28, 2010; doi:10.1152/ajpregu.00498.2009.—In pregnant sheep, maternal-fetal exchange occurs across placental membranes composed of placental cotyledonal and uterine caruncular tissues. Recently, we reported that fetal weights of obese (OB) ewes [fed a diet of 150% of National Research Council (NRC) recommendations] were ∼30% greater than those of control (C) ewes (fed a diet 100% of NRC recommendations) at midgestation (MG), but fetal weights were similar in late gestation (LG). Transplacental nutrient exchange is dependent on placental blood flow, which itself is dependent on placental vascularity. The current study investigated whether the observed initial faster and subsequent slower fetal growth rate of OB compared with C was associated with changes in cotyledonal vascularity and expression of angiogenic factors (vascular endothelial growth factor, fibroblast growth factor-2, placental growth factor, angiopoietin-1 and -2). Cotyledonal arterial diameters were markedly greater (P < 0.05) in OB than C ewes at MG, but arterial diameter of C ewes increased (P < 0.05) from MG to LG, they remained unchanged in OB ewes. Cotyledonal arterial angiogenic factors mRNA and protein expression were lower (P < 0.05) in OB than C ewes at MG and remained low from MG to LG. In contrast, mRNA levels of angiogenic factors in C ewes declined from high levels at MG to reach those of OB ewes by LG. The increase in cotyledonal arterial diameter in early to MG may function to accelerate fetal growth rate in OB ewes, while the decreased cotyledonal arterial angiogenic factors from MG-LG may function to protect the fetus from excessive placental vascular development, increased maternal nutrient delivery, and excessive weight gain.

Maternal obesity and overnutrition affect fetal growth rate and angiogenesis, which are thought to be important in determining fetal size. This study investigated whether these factors are associated with changes in cotyledonal vascularity and expression of angiogenic factors (vascular endothelial growth factor, fibroblast growth factor-2, placental growth factor, angiopoietin-1 and -2) in the ewe. Recent evidence suggests that a ten-
before assignment to the study and were randomly distributed across dietary treatment groups. A BCS on a scale of 1 (emaciated) to 9 (obese) was assigned by 2 trained observers after palpation of the transverse and vertical processes of the lumbar vertebrae (L2 through L5), the ribs and the region around the tail head. BCS is highly related to carcass lipids and has been used to estimate energy reserves available to ewes (40).

Ewes of similar BCS were assigned at random to be fed either a highly palatable diet, at 100% (control, C; n = 20) of National Research Council (NRC) recommendations (12) or 150% (obese, OB; n = 20) of NRC recommendations, as previously reported (16). Experimental diets were delivered on a dry matter basis to meet the total digestible nutrients requirements for maintenance for an early pregnant ewe based on metabolic BW (BW0.75) from 60 days before mating to day 75 or day 135 of gestation. The number of fetuses carried by each ewe was determined by ultrasonography (Asonics Microimager 1000 sector scanning instrument, Ausonics, Sydney, Australia) at day 45 of gestation. All ewes used in this study carried twin fetuses and were necropsied at day 75 (n = 5/dietary group) or day 135 (C; n = 4; OB; n = 6) of gestation. Placentas of both twin fetuses were selected for study. All ewes were weighed weekly, and BCS on a scale of 1 (emaciated) to 9 (obese) were recorded monthly to evaluate changes in body fatness.

At either day 75 or day 135 of gestation, ewes were weighed, sedated with ketamine (22.2 mg/kg body wt), and anesthetized via isoflurane inhalation (1–2%). Ewes were euthanized by exsanguination, while under general anesthesia, and the gravid uterus was recovered. Fetal body weights and crown rump lengths (CRL) were recovered. Fetal body weights and crown rump lengths (CRL) were then averaged. For each arteriole, its area and perimeter were measured, and the arteriole diameter was calculated accordingly. Diameters of all arterioles on one section were averaged.

Immunohistochemistry. Processing of placental sections for immunostaining was accomplished as described above for vascular density measurement. Immunostaining of specific proteins was accomplished following the general immunohistochemistry protocol with the ABC kit previously described by Zhu et al. (53). Briefly, two 5-μm sections per placenta were utilized for staining of each target protein, with another section from the same placenta serving as a negative control. First, tissue sections were deparaffinized and hydrated by routine methods; then antigen retrieval was accomplished through boiling in citrate buffer (pH = 6.0) for 30 min. Nonspecific antigenic sites were blocked with blocking buffer: normal goat serum (Vector Laboratories, Burlingame, CA) diluted in PBS [1.5% (wt/vol) in PBS buffer (pH = 7.0), with 0.05% (wt/vol) Tween 20 for 1 h at room temperature]. Sections were incubated for 2–3 h at room temperature with anti-VEGF (1:50 dilution), anti PLGF (1:20 dilution), or anti-FGF-2 (1:50 dilution) antibody diluted in blocking buffer. Negative controls were incubated only with blocking buffer for the same period of time. The sections were then incubated at room temperature with a biotinylated second anti-rabbit antibody (1:200 dilution) diluted in blocking buffer. After rinsing with PBS, the sections were incubated at room temperature with a peroxidase-conjugated biotin-avidin complex (Vectastain ABC kit; Vector Laboratories) for 30 min at room temperature. The target proteins were revealed by reacting with 3,3-diaminobenzidine color substrate, according to the manufacturer’s instructions (Vector Laboratories), and further counterstained with Harris-modified hematoxylin.

Total RNA extraction and single-strand DNA synthesis. Cot arterioles were pulverized in liquid nitrogen. Total RNA was extracted from COT arteries (50–100 mg) using TRizol reagent (Invitrogen, Carlsbad, CA) treated with DNase I (Qiagen, Valencia, CA), and then purified by RNeasy minicolumn (Qiagen), according to the respective protocols. One microgram of purified RNA of each COT arterial tissue preparation was used to synthesize single-strand DNA using Promega ImProm- II Reverse Transcription System (Promega Biosciences, San Luis Obispo, CA), according to the kit protocol.

Real-time PCR. Genes studied in the real-time PCR experiments are listed in Table 1. All real-time PCR reactions were conducted using a Bio-Rad IQ5 real-time PCR Reaction System (Bio-Rad Laboratories, Hercules, CA). Reactions for each gene were run in duplicate. A temperature gradient PCR reaction was run for all of the primer sets before conducting real-time PCR to determine the optimal annealing temperature for all primer sets. According to gradient PCR, the optimal annealing temperature of all primer sets overlapped at 60°C. Correspondingly, the following protocol was designed and applied to all real-time PCR reactions: step 1) 1 cycle at 95°C for 3 min; step 2) 40 repeat cycles at 95°C for 10 s, following by annealing at 60°C for 30 s; and step 3) 55.0°C–95.0°C, with melting temperature increasing 0.5°C for each 30 s. Fluorescence was detected at both

| Table 1. Primer sequences used in real-time PCR quantification of selected genes |

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
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</table>
| VEGF | F: 5′-GGA AAG TCT GGA CTC TCT GG-3′
| | R: 5′-TAT GTC TCG GCT TCG TGC AG-3′
| PLGF | F: 5′-GCT CTT TTT TGT GGA GAT GA-3′
| | R: 5′-GTA TCA CCG CAC CTT TTT GG-3′
| FGF-2* | F: 5′-CGA CGG CCG GAT GCA C-3′
| | R: 5′-CTG TCT TCT TCT TGA AGT TAT TAT AG-3′
| ANG-1* | F: 5′-AAA TGA AAG GAA GAA CTA CAG GCT TAT TTA T-3′
| | R: 5′-GGA AGA TGG GGC TCT TTT GTT GCT-3′
| ANG-2* | F: 5′-AAA TAG GCA GCA AGC TGA TCG TGA A-3′
| | R: 5′-TCT TGG GCT ATG TAA TAC TAC TGC GCT-3′
| FLT-1* | F: 5′-TGG ATT TCA GGT AGG CTA CGG-3′
| | R: 5′-TCA CGG TGG AAG ACA GCT TCG-3′
| KDR* | F: 5′-CTT AGA GTC GTC CGG GCC GAG-3′
| | R: 5′-GGA AGA TGA GGC TCT TCT GCT TAT GCT-3′
| Tie2* | F: 5′-CGT GCG AGG GCG GAA GAT G-3′
| | R: 5′-TCA GCC TGG TAT TCT TGC TCT TGC-3′
| HIF-1α* | F: 5′-GCG ATC TGG ATA AGG CCT TCT TTG-3′
| | R: 5′-CAG CAG CAT CCA GGA ATT TCC TCT-3′
| 18 s RNA* | F: 5′-AGG CTG CGG CTT AAT TGG AC-3′
| | R: 5′-GAA CTA AGA ACG CGG ATG CA-3′

*Primer sequences were designed according to the references as listed in MATERIALS AND METHODS. F and R, forward and reverse primers, respectively.
step 2 and step 3. Real-time PCR analysis was enabled at step 2, and melt curve data collection and analysis were enabled at step 3. An amplification efficiency of 90–105% is considered to be high in an optimized real-time PCR reaction (7). In our real-time PCR experiments, all reactions reached 100% efficiency. Final data were analyzed through the 2−ΔΔCt method (26), where 18s RNA was used as a reference gene to normalize all the selected gene expression data.

Primers. VEGF and PLGF primers were designed using Primer 3 based on genetic sequence X89506 and AY157708, respectively. FLT-1, KDR, ANG-1, ANG-2, and Tie-2 primers were synthesized according to Redmer et al. (33), while HIF-1α, 18s RNA primers were synthesized, according to Johnson et al. (23) and Lopez-Andreo et al. (27), respectively. All primers were synthesized by Invitrogen (Carlsbad, CA). Their sequences are as listed in Table 1.

Total protein extraction and Western blot analysis. Liquid-nitrogen pulverized COT arterial samples (80–100 mg) were homogenized in a polytron homogenizer, with ice-cold lysis buffer [50 mM Tris-HCl, 100 mM NaF, 1 mM MgCl2, 2.5 mM EDTA (4 Na), 100 mM NaCl, 2% SDS, 1% NP-40, 0.5 M CaCl2, 2 mM Na3VO4, pH = 7.4]. Homogenates were then sonicated and clarified by centrifugation. After centrifugation, the supernatant was mixed with SDS sample loading buffer and heated at 95°C for 5 min. A standard SDS-PAGE run was used to separate proteins followed by the transfer of separated proteins to a nitrocellulose membrane and blocking with primary and secondary antibodies dissolved in 5% (wt/vol) and 2% (wt/vol) skim milk. The membrane was then visualized using ECL Western blotting detection reagents and exposed to film. The density of bands was quantified by using an Imager Scanner II (Amersham Biosciences, Piscataway, NJ) and ImageQuant TL software (Amersham Biosciences). The target gene band density was normalized according to the density of a reference sample, as well as β-actin content in the same samples.

Antibodies. Anti-VEGF antibody (A-20, rabbit IgG, sc-152), ANG-1 antibody (C-19, goat IgG, sc-6320), ANG-2 antibody (C-19, goat IgG, sc-7015), and anti-goat IgG antibody (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PLGF antibody (rabbit IgG, ab9542) was purchased from Abcam (Cambridge, MA). Anti-FGF-2 antibody (rabbit IgG, AB-33-NA) was purchased from R&D Systems (Minneapolis, MN). Anti-β-actin antibody (mouse IgG, A-1978) was purchased from Sigma-Aldrich (St. Louis, MO). Secondary anti-mouse IgG (#7076) and secondary anti-rabbit IgG (#7074) antibodies were both purchased from Cell Signaling Technology (Danvers, MA).

Statistical analysis. When comparing across dietary treatment and day of gestation, data were analyzed as a complete randomized design using GLM (general linear model of Statistical Analysis System) (SAS 9.1, 2002–2003; SAS Institute, Cary, NC). Mean separation was performed using LSMEANS. Student’s t-tests were performed to compare differences of individual measurements between the two dietary groups at either day 75 or day 135 of gestation (Microsoft Excel 2008 for Mac OS). Additional Student’s t-tests were performed to determine differences of a measurement within a dietary group at different stages of pregnancy. Data are presented throughout as means ± SE and are considered significantly different when P < 0.05, while P values between 0.05 and 0.10 were considered trends.

RESULTS

Ewes on the OB diet increased their body weight by ~30% from diet initiation to mating (71.6 ± 3.2 and 92.8 ± 2.9 kg, respectively; P < 0.05) and increased 43% and 52% in body weight from diet initiation to necropsy on day 75 or day 135 of gestation, respectively. In contrast, C ewes, whose body weight was similar to that of OB ewes at diet initiation, exhibited only modest nonsignificant increases in body weight from diet initiation to conception (2.9%), or necropsy on day 75 or day 135 of gestation (5.7% and 7.0%, respectively). Similarly, BCS of OB ewes increased (P < 0.05) from diet initiation to mating (5.0 ± 0.3 and 7.2 ± 0.2, respectively) and had further increased (P < 0.05) to 8.0 ± 0.2 by day 75, and 8.6 ± 0.2 by day 135, while BCS of control ewes remained relatively constant from diet initiation to day 135 of gestation averaging 4.9 ± 0.4.

 Fetuses from OB ewes necropsied on day 75 of gestation were ~30% heavier (P < 0.05) and had greater (P < 0.05) CRL than fetuses gestated by C ewes on day 75 of gestation (Table 2). In contrast, fetal weight and CRL of C and OB ewes on day 135 of gestation were not different from each other. Total placenta number, total placenta weight, and average placenta weight did not differ across the two dietary groups on day 75 of gestation (Table 2). Total placenta number of C ewes on day 135 of gestation tended to be reduced (P = 0.08) compared with day 135 OB ewes or compared with day 75 C and OB ewes. In contrast, total placenta weight and average placenta weight of OB ewes on day 135 were markedly reduced (P < 0.05) compared with that of C ewes on day 135, as well as C and OB ewes on day 75 (Table 2). The fetal-to-placental weight ratio, an index of placental efficiency, was greater in OB than C ewes on both day 75 and day 135 of gestation (P < 0.05).

COT arteriole numbers per placenta were similar for C and OB ewes on day 75, but vessel diameters were greater (P < 0.05) in OB than C ewes (Fig. 1). On day 135 of gestation, while COT arteriole numbers per placenta remained similar between C and OB ewes, vessel numbers in both dietary

Table 2. Fetal and maternal data from control ewes fed 100% of the NRC-recommended diet, and obese ewes fed 150% of the NRC-recommended diet on both day 75 and day 135 of gestation

<table>
<thead>
<tr>
<th>Day 75 of Gestation</th>
<th>C</th>
<th>OB</th>
<th>Day 135 of Gestation</th>
<th>C</th>
<th>OB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td></td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Number of twin pregnancies</td>
<td>5</td>
<td>5</td>
<td>502.45 ± 163.45</td>
<td>4827 ± 169.15</td>
<td></td>
</tr>
<tr>
<td>Fetal weight, g</td>
<td>185.7 ± 6.95</td>
<td>234.4 ± 6.68</td>
<td>58.1 ± 0.75</td>
<td>57.1 ± 0.86</td>
<td></td>
</tr>
<tr>
<td>Crown rump length, cm</td>
<td>17.3 ± 0.50</td>
<td>19.6 ± 0.49</td>
<td>72.8 ± 3.55</td>
<td>84.3 ± 4.65</td>
<td></td>
</tr>
<tr>
<td>Number of placentomes</td>
<td>88.6 ± 4.85</td>
<td>93.4 ± 5.00</td>
<td>623.3 ± 15.85</td>
<td>486 ± 14.25</td>
<td></td>
</tr>
<tr>
<td>Total placenta weight, g</td>
<td>709.5 ± 38.95</td>
<td>654.6 ± 37.65</td>
<td>17.2 ± 0.65</td>
<td>11.6 ± 0.65</td>
<td></td>
</tr>
<tr>
<td>Average placenta weight, g</td>
<td>16.3 ± 0.96</td>
<td>14.4 ± 1.14</td>
<td>8.1 ± 0.35</td>
<td>10 ± 0.46</td>
<td></td>
</tr>
</tbody>
</table>

NRC, National Research Council; C, control; OB, obese. a,b,c,dValues are means ± SE within a row when superscripted letters differ (P < 0.05). e,fValues are means ± SE within a row when superscripted letters differ (P < 0.10).
MATERNAL OBESITY ALTERS COTYLEDONARY VASCULARITY

The mRNA level of VEGF, FGF-2, PLGF, ANG-1, and ANG-2 in OB ewe COT arterial tissue were decreased 64.2%, 49.9%, and 64.2% of gestation, respectively. In contrast to the COT, however, CAR arterioles diameters were similar in C and OB ewes remained unchanged over the same interval (Fig. 1). The CAR arteriole numbers followed a pattern similar to that of the COT. The numbers of CAR arterioles were similar in C and OB ewes on days 75 and 135, with vessel numbers in both groups increasing (P < 0.05) over this period and averaging 4.02 ± 0.98 and 7.39 ± 1.13 per mm², respectively. In contrast to the COT, however, CAR arteriole diameters were similar for C and OB ewes and remained relatively constant from day 75 to day 135, averaging 108 ± 13.70 and 83.73 ± 15.76, respectively.

The mRNA level of VEGF, FGF-2, PLGF, ANG-1, and ANG-2 in OB ewe COT arterial tissue were decreased 64.2%, 49.9%, and 64.2% of gestation, respectively. In C ewes, the significant increase in diameter of the COT arteries of OB ewes (Fig. 3, A and B), while ANG-1 expression tended to be lower (P = 0.08). Also, in agreement with the real-time RT-PCR data, protein expression of VEGF, PLGF, FGF-2, and ANG-1 of C and OB ewes were similar on day 135 of gestation (Fig. 4, A and B). In contrast, protein expression of ANG-2 was increased in COT arteries on day 135 in OB vs. C ewes (P < 0.05). In Western blot analysis, VEGF and PLGF were detected at ~28 kDa in our sheep tissue, exactly the same size as the positive control (sc-2210; Santa Cruz Biotechnology, Santa Cruz, CA). The PLGF antibody, according to the product description, does not cross react with VEGF protein. FGF-2 was detected at ~25 kDa, slightly higher than the protein size described by the company, probably due to posttranscriptional modification, such as glycosylation. ANG-1 and ANG-2 were detected at ~55 kDa.

To relate differences in angiogenic factor expression found in COT arterial tissue on day 75 between C and OB females to the surrounding COT tissues, COT tissue from the placentomes supplied by these arteries were evaluated for protein expression of VEGF, PLGF, FGF-2, ANG-1, and ANG-2. As depicted in Fig. 5, A and B, protein expression of PLGF, ANG-1, and ANG-2 were found to be higher (P < 0.05), and VEGF tended to be higher (P = 0.09) in COT tissue from OB vs. C ewes. Protein expression of FGF-2 was similar in COT tissues of C and OB ewes.

Fig. 6 illustrates endothelial localization of VEGF, PLGF, and FGF-2 in cotyledonary blood vessels of placentomes collected from day 75 (Fig. 6A) and day 135 (Fig. 6B) of gestation, respectively. In nonvascular placental tissues, VEGF, PLGF, and FGF-2 were mainly localized in the cytoplasm and nucleus of trophoderm cells at the fetal-maternal interface. The nuclei of binuclear cells were stained as the trophoblastic cells due to the Harris-modified hematoxylin stain in the maternal caruncular tissue. However, the cytoplasmic area of those binuclear cells failed to be stained with any of the three angiogenic factors, indicating that the three angiogenic factors were not expressed in those binuclear cells.

DISCUSSION

To the authors’ knowledge, this is the first large animal model established to study the impacts of increased maternal calorie intake leading to preconceptional maternal obesity and continued excessive weight gain during pregnancy on fetal and placental growth, and placental angiogenic factor expression and vascularization. The sheep is one of the most commonly and extensively investigated precocial species in biomedical studies on human pregnancy (17, 19, 25, 32). These data demonstrate a markedly (~30%) increased fetal weight in OB vs. C ewes at midgestation, indicating a greater fetal growth trajectory in OB females during the first half of gestation. This increase in fetal growth was associated with a marked increase in COT arteriole diameters compared with those of C ewes. Arterioles are the most highly regulated blood vessels in the body, and contribute the most to overall blood pressure. Arterioles respond to a wide variety of chemical and electrical messages and are constantly changing size to speed up or slow down blood flow through the capillary bed (28). In C ewes, the significant increase in diameter of the arterioles in COT tissue from day 75 to day 135 of gestation would be expected to dramatically increase COT blood flow and hence promote nutrient delivery to the fetus to meet the requirement of rapid fetal growth during the second half of gestation. The
failure of COT arterioles to increase in diameter coupled with a reduction of capillary growth and proliferation due to reduced COT angiogenic factor expression in OB ewes may have resulted in the slowed fetal growth observed in this group compared with C ewes.

Downregulation of growth factors and/or increases in inflammatory factors in the fetal circulation is known to slow placental and fetal growth and development during gestation (11, 52).

While numbers of COT arterioles were similar in C and OB ewes on day 75, arteriole diameters were \( \sim 35.5\% \) greater in OB ewes. If we apply Poiseuille’s Law (see right), which describes the variables controlling flow through the vasculature, the blood flow through COT tissue of OB ewes was 337\% greater than in C ewes, if length, pressure, and blood viscosity remain constant.

Poiseuille’s Law:

\[
\text{Volume flow rate} = \frac{(\text{Pressure difference})(\text{Radius})^4}{8(\text{Viscosity})(\text{Length})}
\]

Such an increase in blood flow would provide a mechanism for the observed fetal overgrowth in OB ewes at day 75 of gestation.

Fig. 2. A: real-time PCR quantification of selected angiogenic factors mRNA levels in COT arterial tissue from both C and OB ewes at day 75 of gestation (C, \( n = 5 \); OB, \( n = 5 \)) and day 135 of gestation (C, \( n = 4 \), OB, \( n = 6 \)). B: receptors of selected angiogenic factors and hypoxia inducible factor-1\( \alpha \) (HIF-1\( \alpha \)) mRNA levels in COT arterial tissue from both C and OB ewes at day 75 of gestation (C, \( n = 5 \); OB, \( n = 5 \)) and day 135 of gestation (C, \( n = 4 \), OB, \( n = 6 \)). a,b,cValues are expressed as means \( \pm \) SE, where superscripted letters that differ show significant difference (\( P < 0.05 \)). d,eValues are expressed as means \( \pm \) SE within a treatment group where superscripted letters that differ show significant difference (\( P < 0.10 \)). VEGF, vascular endothelial growth factor; FGF-2, fibroblast growth factor; PLGF, placenta growth factor; FLT-1, FMS-like tyrosine kinase-1; KDR, kinase insert domain receptor.

Fig. 3. A: Western blot measurement of selected protein expression in COT arterial tissue of both C (\( n = 5 \)) and OB (\( n = 5 \)) ewes at day 75 of gestation. Representative Western blot bands to VEGF, PLGF, FGF-2, ANG-1, and ANG-2, and the reference protein \( \beta \)-actin are shown. B: statistical analysis on Western blot result of the five proteins in A. Values are expressed as means \( \pm \) SE; *\( P < 0.05 \). Trend toward difference, §\( P = 0.08 \).
gestation. Alternatively, the increased blood flow might represent a response to the increased growth rather than a cause. Similar explanations would apply to the interesting continuation of C ewes COT arterioles to increase ~44.9% in diameter ($P < 0.05$), while OB ewes COT arterioles didn’t show an increase in diameter from day 75 to day 135 of gestation. Again applying Poiseuille’s Law, there is a potential 44.4% increase in blood flow through COT tissue from day 75 to day 135 in C ewes if length, pressure, and blood viscosity remain constant. An increase in blood flow of this magnitude would have a markedly increased nutrient delivery to the fetus, thereby providing for the well-documented acceleration in fetal growth in C ewes that occurs during the second half of gestation (5). This increased blood flow in fetuses from C ewes explains the observation of similar body weights and CRL in gestation (5). This increased blood flow in fetuses from C ewes has a markedly increased nutrient delivery to the fetus, thereby providing for the well-documented acceleration in fetal growth (135) in the maternal caruncle from day 75 to day 135 of gestation. Alternatively, the increased blood flow might represent a response to the increased growth rather than a cause. Further, the CAR arterioles exhibited markedly greater diameters than COT arterioles on both day 75 and day 135 of gestation.

Our observations are in agreement with Catalano et al. (10), who reported that fetuses of obese and lean women exhibit similar weights in late-gestation (37–40 wk). Evidence suggesting an accelerated fetal intra-uterine growth rate during first half of gestation followed by a reduced fetal growth rate during the second half of gestation in OB vs. C ewes is suggested by the ratio of brain weight to fetal body weight. At midgestation, fetal brain weight was significantly higher in fetuses of OB ewes than C ewes (7.13 ± 0.19 g and 6.20 ± 0.18 g, respectively, $P < 0.05$). However, the ratio of brain weight to whole body weight was markably lower in OB fetuses than C fetuses (3.02% ± 0.13% and 3.34% ± 0.07%, respectively, $P < 0.05$), suggesting a greater increase in fetal body mass than fetal brain weight in OB fetuses. In contrast, in late gestation, fetal brain weight (C fetus, 58.75 ± 1.06 g; OB fetus, 57.80 ± 1.15 g, $P > 0.1$) and brain/whole fetal body weight (C fetus, 1.17% ± 0.05%; OB fetus, 1.22 ± 0.02%, $P < 0.1$) were similar for OB and C group, suggesting a reduction in growth rate of OB vs. C fetuses from midgestation to late gestation.

As previously discussed, the COT capillary bed grows primarily by branching angiogenesis throughout the last two-thirds of pregnancy, resulting in a large (>10 fold) increase in capillary area density, accompanied by a decrease in capillary size (44). This marked increase in capillary numbers in the COT tissue results in a simultaneous increase in umbilical blood flow and facilitates transplacental exchange. These observations help to explain why the proportion of nutrients and oxygen transported to the fetus increases by 2- to 4-fold from midgestation to late gestation, a requirement for keeping pace with fetal growth (14, 36). While many factors have been implicated in mediating placental angiogenesis, VEGF, PLGF, FGF-2, ANG-1, and ANG-2, as well as their receptors appear to play a major role (35). Throughout the last two-thirds of gestation in the sheep, COT tissue expression of these angi-
Fig. 6. Immunohistochemical staining (brown) of VEGF, PLGF, and FGF-2 in placentomal tissues on day 75 of gestation (A) and day 135 of gestation (B); nuclei are counterstained with hematoxylin (blue). I–III, VEGF (A and B); IV–VI, PLGF (A and B); VII–IX, FGF-2 (A and B); X, negative control incubated with blocking buffer with no primary antibody (A and B). M, maternal caruncular tissue; F, fetal COT tissue; FB, fetal blood vessel; MB, maternal blood vessel; T, trophoblast cells; BNC, binucleate cells; E, Endothelial layer. An asterisk (*) denotes red blood cells.
genetic factors is positively correlated with measures of angiogenesis, including capillary area density, capillary number density, and capillary surface density (35). Further, both VEGF and FGF-2 have been implicated in regulating uterine blood flow through increases in endothelial nitric oxide production, a major local vasodilator (4, 20, 37).

In this study, we report decreases in VEGF, PLGF, FGF-2, ANG-1, and ANG-2 mRNA and protein levels on day 75 of gestation in OB ewe COT arteries. Further, we observed that HIF-1α, a compound known to stimulate angiogenic factor expression (41, 42) also tended to be reduced in COT arteries of OB vs. C ewes on day 75. These data suggest the presence of a fetal/maternal signal-reducing COT arterial angiogenic factor expression and vascular growth in the face of maternal overnutrition. This would be expected to reduce nutrient delivery to the fetal maternal interface, slowing fetal growth rate and preventing fetal overgrowth and dystocia at the time of birth. These data are consistent with those of Redmer et al. (33), who reported a reduction of VEGF, ANG-1 and ANG-2, and FLT-1 mRNA expression in whole placentomes recovered from overnourished adolescent ewes on day 81 of gestation compared with those recovered from adolescent ewes fed only to basic requirements. These data, however, were not confirmed at the protein level in that study (33). Further, these authors (33) reported that uterine arterial blood flow was reduced by 50% on day 90 of gestation in these adolescent overnourished ewes compared with adolescent ewes fed to basic requirements. They concluded that these decreases in angiogenic factor expression may be part of the causal mechanisms since they preceded any reduction in placental wet weight, which may constitute an early defect in these adolescent overnourished pregnancies. Because mRNA and protein expression of angiogenic factors were markedly reduced in COT arterial tissues of OB vs. C ewes on day 75, it is possible that capillary growth and blood flow were already slowing in the COT of OB ewes, potentially beginning to impact nutrient diffusion into the fetal compartment. Establishment of the timing and cause of the decrease is an important goal of future studies.

From midgestation to late gestation, a slowing of fetal growth in OB vs. C ewes was observed, such that by day 135, fetuses of C and OB ewes were similar in size. These data are in agreement with Catalano et al. (10), who reported that late-gestation fetuses (37–40 wk) of obese and lean women were similar in size in late-gestation (37–40 wk). These data suggest a slowing of fetal growth in OB vs. C ewes during the second half of gestation. While the numbers of COT arterioles doubled from day 75 to day 135 in both C and OB ewes, arteriolar diameter increased significantly in C ewes, while remaining similar in OB ewes. These data are consistent with the concept that at a time when uteroplacental blood flow increases three-fold in normal pregnancies (30), the placenta of OB ewes are limiting uteroplacental blood flow and fetal nutrient delivery. Reductions in fetal-placental flow in the presence of maternal overnutrition in our study would be consistent with the observations of Wallace et al. (48), who reported that late-gestation umbilical blood flow was lower per kilogram fetus, in overnourished adolescent ewes than adolescent ewes fed to requirements, suggesting a reduced perfusion of the placenta by the fetus. While we used mature ewes in the present study and Wallace et al. (2002) used adolescent ewes, the accelerated increase in the body weight of pregnant ewes on the obesogenic diets in both studies may have resulted in similar changes placental vascularity, but additional studies are required to confirm this hypothesis. Lang et al. (24) reported that a chronic reduction in uterine blood flow from day 113 to day 138 of gestation in the ewe decreased fetal body weight by 32%, which is similar to the relative reduction seen in OB vs. C ewes from midgestation to late gestation in this study. Further, these researchers (24) reported that this chronic reduction in uterine blood flow decreased relative placental weight by 34%, which again is similar to the differences observed in total placental weight between OB and C ewes in the present study on day 135, which averaged ~28%. In another recent study, Redmer et al. (34) observed a similar decrease (P < 0.05) in placental weight at day 130 of gestation in the overnourished pregnant adolescent sheep vs. control-fed pregnant adolescent sheep. It is well accepted that placental angiogenesis is one of the earliest events in placental development and is required to supply nutrients and oxygen for these growing tissues (39). The dramatically increased COT arteriole diameters from midgestation to late gestation reported in this study, coupled with increased capillary diameters and densities (S. P. Ford et al., unpublished observations) in C ewes vs. OB ewes would be expected to further increase blood flow into the COT vascular bed, promoting the growth of the placentome itself. The significant increase in placental efficiency (fetal weight/placental weight ratio) of OB vs. C ewes at midgestation, results from fetal overgrowth, while at late gestation, it results from decreased placental weight. These data suggest the presence of a fetal signal reducing COT blood flow and thus placental growth and nutrient delivery to the fetal compartment in the face of elevated maternal feed intake and circulating nutrient concentrations. The term coined by Lang et al. (24) to explain the decrease in placental weight resulting in response to this chronic reduction in uterine blood flow was “blood flow-mediated adaptive regression.” This concept is supported by the changes in COT angiogenic factor expression and vascularity observed in this study. While COT arterial tissue angiogenic factor (VEGF, FGF-2, ANG-1, and ANG-2) expression decreased progressively from day 75 to day 135 in C ewes, these same angiogenic factors remained low and constant from midgestation to late gestation in OB ewes.

A similar early gestational acceleration in fetal growth followed by a reduced growth trajectory from midgestation to late gestation has recently been described by Redmer et al. (34) in an overnourished adolescent ewe model. Compared with control fed adolescent ewes, these researchers observed an 11% increase in fetal weight in overnourished adolescent ewes by day 90 of gestation, followed by a 20% decrease by day 130 of gestation. Similar to the results presented here for the mature multiparous ewes, pregnant overnourished adolescent ewes exhibit a reduction in placental mass by late gestation, but unlike the current study, which overnourished mature ewes, their adolescent overnourished ewes gave birth prematurely to low-birth-weight lambs (47, 48). In contrast, using mature ewes, previous research in our laboratory demonstrated similar birth weight between C and OB ewes, which averaged 5,320 ± 470 and 6,000 ± 321 g, respectively (16). A significant decrease in placentome numbers has also been reported in adolescent overnourished ewes vs. control fed adolescent ewes on day 134 (48) and at birth (46). The authors of this report
concluded that this decrease was due to a flow-induced atrophy or regression of a portion of the placentomes during the second half of gestation. No similar decrease in placentome numbers was observed in the present study, where numbers of placentomes of OB ewes tended to be greater than C ewes on day 135. Additionally, previous research in our laboratory demonstrated similar placentome numbers between C and OB ewes at term, which averaged 81 ± 4 and 78 ± 8, respectively (n = 5/group; S. P. Ford et al., unpublished observations). These data suggest that in contrast to overnourished adolescent ewes, mature obese overnourished ewes slowed fetal and placental growth in late gestation without significant reductions in placentome number or birth weight. This difference may result from a greater ability of older mature ewes, as used in this study, than adolescent ewes to meet or exceed their own nutrient requirements, while at the same time partitioning adequate nutrients for fetal growth. Our study and that conducted with overnourished adolescent ewes cannot be directly compared, however, since the ewes in our study were already obese at the time of conception, while those in the adolescent ewe study only became obese several weeks into gestation (49).

The lack of any differences in CAR arteriole numbers and diameters between C and OB ewes at either midgestation or late gestation is consistent with the concept that differences in uterine artery arteriogenesis played a minor role, if any, in the group differences in placental vascularity and fetal growth reported here. This is supported by Gassman et al. (18), who reported that uterine arteriogenesis during a gestation is largely irreversible, and thus after several pregnancies, does not play a major role in pregnancy outcome (18).

Regulation of fetal growth is largely a balance between fetal nutrient demand determined by the individual fetal genetic growth potential and the intrauterine environment, in which the maternal to fetal nutrient supply is a major factor. Factors that determine maternal to fetal nutrient supply include maternal nutrition and metabolism, the transplacental concentration gradient, uteroplacental blood flow, placental size, and placental transport capabilities (31, 54). Zhu et al. (54), using the same experimental paradigm utilized in this study, reported that maternal blood glucose and insulin concentrations were markedly higher in OB ewes and fetuses than C ewes and fetuses at midgestation. Jansson et al. (22) summarized evidence suggesting that gestational diabetes mellitus-induced upregulation of placental nutrient transporter activity, with an associated increase in fetal/neonatal size, may result from elevated insulin concentrations in maternal blood. Insulin has been reported to increase placental glucose transport capacity (13) and System A (neutral amino acid transport) activity (21) in vitro.

**Perspectives and Significance**

These data demonstrate for the first time in a large animal model that prepregnancy maternal obesity resulting from dietary excess causes an initial accelerated fetal growth rate to midgestation, followed by a markedly reduced fetal growth trajectory to term compared with animals fed to basic requirements. By midgestation, COT arteriole diameters of OB conceptuses were markedly larger than those of C conceptuses, potentially increasing COT blood flow and nutrient delivery from mother to fetus, and increasing fetal weight. The marked decrease in expression of key angiogenic factors observed at midgestation in the COT arteries of OB vs. C conceptuses would be expected to decrease capillary proliferation in the COT, slowing fetal growth rate. The signal whereby placental vascular growth is suppressed in the OB placenta is, at present, unknown, but leads to normal birth weights at term in these OB animals. Results from our ovine model are similar to outcomes of human pregnancies, where infants from obese mothers often exhibited macrosomia in gestation but are born at normal weights. These infants, however, exhibit a marked increase in adiposity at term, which is associated with insulin resistance and obesity in postnatal life. The similarity of pregnancy outcomes in our obese sheep model and the human data from obese pregnancies suggest common mechanisms, which could potentially lead to a better understanding of the specific control mechanisms involved.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**REFERENCES**


