Ontogeny of endothelial nitric oxide synthase mRNA in an ovine model of fetal and placental growth restriction

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OBJECTIVE: To determine: 1) placental eNOS mRNA concentration across gestation in normal ovine pregnancy and in an ovine model of intrauterine growth restriction (IUGR), and 2) placental eNOS protein concentration in early ovine pregnancy.

STUDY DESIGN: A total of 24 sheep were studied with 12 ewes placed in hyperthermic (HT) conditions to induce IUGR and 12 were kept in control conditions. HT and control animals underwent euthanasia at 3 developmental time points (55, 95, & 130 days gestational age; dGA) in ovine placental & fetal development.

RESULTS: Compared to controls, HT pregnancies showed 1) no differences in fetal weights at 55 dGA and 95dGA with significant reductions at 130 dGA, 2) significantly smaller placentae at 95 and 130 dGA with a trend for a reduction at 55 dGA, 3) significant decreases in cotyledon eNOS mRNA at 95 and 130 dGA, 4) a significant increase in caruncle eNOS mRNA expression at 130 dGA, 5) significant increase in eNOS protein in the caruncle, but not in the cotyledon at 55 dGA.

CONCLUSION: Placental eNOS concentration is transcriptionally regulated at mid-gestation, while additional post-transcriptional regulation is also involved during early and late gestation in this model of placental and fetal growth restriction.

Key words: endothelial nitric oxide synthase, IUGR, ontogeny


I

trauterine growth restriction (IUGR) is a significant complication of pregnancy associated with an increased risk for morbidity and mortality.1,2 Placental insufficiency is the most common cause of IUGR,3,4 which is characterized by altered expression of vasoactive factors including nitric oxide (NO).5 Endothelial nitric ox-

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aterials and Methods

Animals and tissue preparation

This study was approved by the University of Colorado Health Sciences Center Animal Care and Use Committee. Animal care was performed as previously described by Galan et al.11 A total of 24 sheep were studied, with 12 of these placed in HT conditions beginning at 40 dGA (term = 147 dGA) to induce IUGR and 12 animals placed in thermoneutral conditions beginning at 40 dGA (term = 147 dGA) to induce control conditions. HT and control animals underwent euthanasia at 3 developmental time points (55, 95, & 130 days gestational age; dGA) in ovine placental & fetal development.

While alterations in eNOS abundance may be a determinant of altered blood flow and vasoreactivity in our IUGR model, the mechanism for the altered eNOS protein concentrations has not been examined.12,13 In addition, eNOS protein concentrations have only been assessed at midgestation and near-term. In the present study, we determined the following: 1) placental eNOS mRNA ontogeny across gestation, and 2) eNOS protein concentration during early gestation. This model is characterized by reduced umbilical and uterine flows, fetal hypoxia, reduced placental amino acid and glucose transfer, abnormal placental resistance, and systemic hypertension.11,14-16 We hypothesize that eNOS mRNA concentration will be decreased at midgestation (95 days of gestation; dGA) and increased near-term (130 dGA), thus matching the previously reported eNOS concentration data that we have previously reported. However, we do not expect changes in placental eNOS mRNA or protein during early gestation (55 dGA).
conditions as controls. HT and control animals underwent euthanasia at 3 time points representing early gestation (55 dGA), when placental growth rate is maximal, midgestation (95 dGA), when placental size is at its maximum, and near-term (130 dGA), where the rate of fetal growth is greatest. Animals were equally divided among each time point so that there were 4 animals per HT and control group for each gestational age. At necropsy, the fetal and placentome weights were recorded. Placentomes were separated into the maternal (caruncle) and fetal (cotyledon) components, and frozen in liquid nitrogen for further analysis. A time line for these studies is shown in Figure 1 depicting hyperthermic exposure and the timing of the necropsies as they correlate to fetal and placental development.

RNA extraction
Total RNA was extracted from the collected tissues using the TRI REAGENT method. One hundred mg of each tissue were homogenized in 1 mL of TRI REAGENT (Sigma, St Louis, MO). After homogenization, samples were centrifuged, chloroform extracted, precipitated, and washed in cold 75% ethanol before being further purified with a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). To quantify the RNA, sample absorbance was measured at 260 and 280 nm using a GE Healthcare Ultraspec 4300 Pro UV-VIS spectrophotometer (GE Healthcare, Piscataway, NJ).

cDNA synthesis
CDNA was produced through reverse transcription using the First-Strand cDNA Synthesis protocol from the SuperScript III kit by Invitrogen (Invitrogen, Carlsbad, CA). Five µg of total RNA was mixed with 50 µmol/L of oligo (dT) primers, 10 mmol/L dNTP mix and DEPC-treated water. Next, the samples were incubated at 65°C for 5 minutes and then placed on ice for at least 1 minute. Ten µL of cDNA Synthesis mix (10× RT buffer, 25 mmol/L MgCl2, 0.1 mol/L DTT, RNase out, and SuperScript III RT) was added to each sample and then incubated at 50°C for 60 minutes. Reactions were terminated by incubation at 70°C for 15 minutes. RNase H (1 µL) was added to each sample and then incubated at 37°C for 20 minutes. The samples were stored at −20°C until needed.

Reverse transcriptase-PCR and sequencing of eNOS
RT-PCR was performed using cDNA generated as explained. Ovine eNOS forward (5′-TGC ATG AGA TGG AGA GCA AAG GGC −3′) and ovine eNOS reverse (5′-ATG TCC TCG TGA TAG CGT TGC TGA −3′) primers were used at an annealing temperature of 60.3°C during RT-PCR. The eNOS PCR product (391 bp) was purified using a QIAquick PCR Purification kit and sequenced for authenticity. The 391 bp RT-PCR product was 99% identical to the published ovine eNOS nucleotide sequence (DQ015701). Our eNOS cDNA exhibited no significant homology with nNOS (U766739) or iNOS (AF223942) sequences confirming the specificity of our eNOS product.

Real time PCR
Quantitative real time PCR was used to quantify mRNA concentrations in our samples. Ten ng of each sample cDNA was subjected to real time PCR using our eNOS forward/reverse primers (same as above), and compared to a standard curve generated by known quantities of eNOS cDNA to determine starting quantity. To normalize our eNOS data, sample cDNA were subjected to real-time PCR using primers (forward 5′-TCA ACC AGG TGG AGA TCA ACG −3′ and reverse 5′-TGC TTT ACG GCC TTG TAG GTG −3′) for ribosomal protein S15, and a standard curve of known quantities of S15 cDNA. The amplification efficiencies were 98% and 99% for eNOS and S15, respectively.

Western blot analysis
Cotyledons and caruncles of 55 dGA pregnancies were homogenized in protein lysis buffer (10 mmol/L of PMSF, 10 mmol/L of Na3VO4, 1× triton X-100, 150 mmol/L of NaCl, 20 mmol/L Tris Base, 5 mmol/L AEBSF, 5 mmol/L EDTA, 10 mmol/L of E-64, 10 nm Leupeptin, and 10 ng/mL Aprotinin). Protein tissue lyastes (50 µg) were separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with a mouse HRP-conjugated antibody against eNOS (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were incubated with chemiluminescent substrate (Pierce, Rockford, IL) for 5 minutes and exposed to x-ray film. To determine loading consistencies, each membrane was stripped of antibodies and reincubated with antibody against mouse beta-actin (dilution 1:4000) (MP Biomedicals, Aurora, OH). The abundance of these proteins was quantified by densitometry.

FIGURE 1
Time-line diagram of the study timepoints as they correlate to fetal and placental development

<table>
<thead>
<tr>
<th>Control Conditions</th>
<th>HT Conditions</th>
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<tbody>
<tr>
<td>dGA=days gestational age</td>
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</tr>
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</table>

HT Group Control Group
dGA 40 55 95 120 130

Enter HT 1st Necropsy 2nd Necropsy Exit HT 3rd Necropsy

[46x130]4300 Pro UV-VIS spectrophotometer nm using a GE Healthcare Ultrospec absorbance was measured at 260 and 280 nm using a GE Healthcare Ultraspec 4300 Pro UV-VIS spectrophotometer (GE Healthcare, Piscataway, NJ).

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Statistical analysis

Data are presented as mean ± SE. Comparisons were made between control and HT pregnancies for fetal and placental weights, eNOS mRNA, and protein concentrations within a given tissue (cotyledon or caruncle) and gestational age (55, 95, or 130 dGA). The F-test was used to assess equality of variance and the appropriate corresponding t test was used to compare the endpoints between groups statistically. P ≤ .05 was considered significant.

Results

There was no difference in fetal weights between HT and control pregnancies (Figure 2) at 55 dGA (29 ± 2.3 g vs 32 ± 2.6 g; P = .22) or 95 dGA (682 ± 205 g vs 715 ± 11 g; P = .79). However, as previously reported, HT-induced IUGR pregnancies demonstrated significant reductions in fetal weight compared to controls near-term (1.8-fold; 1718 ± 433 g vs 2914 ± 201 g; P ≤ .008). At 55 dGA (Figure 3), HT pregnancies exhibited a trend towards a smaller placenta (1.3-fold; 135 ± 3 g vs 181 ± 20 g; P = .053), and by 95 dGA this trend became statistically significant (2.4-fold; 186 ± 18 g vs 440 ± 50 g; P = .003). The difference in placental weights persisted near-term (2.0-fold; 169 ± 43 g vs 349 ± 21 g; P ≤ .004).

Figure 4 presents eNOS mRNA concentrations at each gestational age sampled for the fetal cotyledon. At 55 dGA, cotyledon eNOS mRNA concentration was not impacted by HT treatment. However, at 95 dGA there was a significant reduction (1.7-fold; P = .04) in HT fetal cotyledon eNOS mRNA concentration, and this reduction was more pronounced near-term (18.8-fold; P = .03).

Similar to the fetal cotyledons, at 55 dGA there was no difference due to treatment in maternal caruncle eNOS mRNA concentration (Figure 5), and by 95 dGA there was a trend (3.3-fold; P = .052) for reduced mRNA concentrations in caruncles derived from HT pregnancies. In contrast, near-term there was a significant increase (5.5-fold; P ≤ .008) in maternal caruncle eNOS mRNA concentration in IUGR pregnancies.

Figure 6 presents the Western blot analysis of eNOS at 55 dGA. Similar to the mRNA concentration data (Figure 4) there was no effect of treatment on cotyledon eNOS concentrations. However, in contrast to the maternal caruncle mRNA concentration data (Figure 5), the concentration of eNOS was significantly increased (P < .04) by HT treatment at 55 dGA.

Comment

Alterations in eNOS regulation in placental and vascular tissues may lead to reduced uterine-umbilical blood flows, increased placental flow resistance, and systemic hypertension in growth restricted pregnancies. Our previous studies of eNOS concentrations in our ovine IUGR model led us to explore 2 primary objectives representing new information and allowing a more complete eNOS expression profile across gestation. In our previous studies, we found cotyledon eNOS concentration to be decreased at

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95 dGA but increased at 130 dGA when the fetus is known to be hypoxic. In this study, we completed the eNOS concentration profile by assessing eNOS at 55 dGA. We found no differences between HT and control pregnancies suggesting that at least early HT exposure does not impact the cotyledonary (fetal side) eNOS concentration. In contrast, 55 dGA caruncle eNOS was increased in the HT pregnancies, suggesting perhaps that the early effects of HT is to enhance eNOS on the maternal side of the placenta.

In order to gain knowledge of the underlying mechanism of placental eNOS regulation, we determined eNOS mRNA concentrations in placental cotyledonary and caruncular tissues at different gestational ages (55 dGA, 95 dGA, and 130 dGA). When comparing HT and control groups, 55 dGA eNOS mRNA concentration data did not indicate differences for either the cotyledon or caruncle tissue in our IUGR animals compared to our controls. When compared with eNOS protein at this gestational age, we also observed no significant difference due to treatment. In contrast, there was a significant increase observed for caruncular eNOS protein at 55 dGA, suggesting posttranscriptional regulation of eNOS in early gestation. At midpregnancy (95 dGA), eNOS mRNA concentration was significantly decreased in cotyledons from IUGR pregnancies, and there was also a trend for a decrease in the caruncle eNOS mRNA concentrations. Near-term (130 dGA) eNOS mRNA was reduced in the cotyledon and increased in the caruncle. The reason for the difference between cotyledon and caruncle eNOS mRNA concentrations at 130 dGA, as a function of IUGR, is not readily apparent. Hypoxia is known to increase eNOS mRNA and protein in the lungs. Interestingly, other reports have shown eNOS mRNA to be decreased in endothelial cells in the rat under hypoxic conditions. Thus, hypoxia appears to have different effects depending on the tissues, species, and experimental conditions. It is known that during early gestation embryos and placenta exist in a hypoxic environment and the HT-IUGR fetuses are hypoxic at birth; thus, hypoxia may be the stimulus for increased eNOS mRNA and protein in the HT placenta at this time point. 13

When placental eNOS mRNA is compared to the previously published protein concentrations at the same time point in our model, a significant decrease in cotyledon eNOS mRNA and a decrease in caruncle eNOS mRNA matched the reductions in eNOS at 95dGA in these tissues. This suggests that eNOS in these tissues appears to be transcriptionally regulated at midgestation in the HT ovine IUGR model. At 130 dGA, placental eNOS mRNA showed differential expression in the IUGR pregnancies with a decrease in the cotyledons and an increase in the caruncles. The increase in caruncle (maternal placental component) eNOS mRNA concentration matches prior studies showing an increase in caruncle eNOS concentration, suggesting transcriptional regulation for this protein. In contrast, concentrations of mRNA and protein are discordant in the cotyledon (fetal side), suggesting a posttranscriptional or translational control of eNOS synthesis.

With the exception of the near-term cotyledon, we conclude that eNOS concentration is transcriptionally regulated in the placenta of HT exposed ovine pregnancies. The posttranslational mechanisms of regulation of eNOS in the near-term cotyledon (fetal side) are unknown and remain to be defined. The profile of eNOS mRNA concentration in the placenta of normal and IUGR ovine pregnancies has been outlined in this study and represents the first report of placental eNOS mRNA concentration across gestation in the ovine placenta.

REFERENCES