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# Developmental Changes in Nitric Oxide Synthesis in the Ovine Placenta[1]

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## ABSTRACT

**Nitric oxide (NO), synthesized from L-arginine by NO synthase (NOS), is a key regulator of placental angiogenesis and growth during pregnancy. However, little is known about placental NO synthesis associated with ovine conceptus development. This study was conducted to test the hypothesis that placental NO synthesis is greatest during early gestation. Columbia cross-bred ewes were hysterectomized on Days 30, 40, 60, 80, 100, 120, or 140 of gestation (n = 4 per day) to obtain placentomes, intercotyledonary placenta, and intercaruncular endometrium. Tissues were analyzed for constitutive NOS (cNOS) and inducible NOS (iNOS) activities, NO synthesis, tetrahydrobiopterin (BH4) and NADPH (essential cofactors for NOS), and GTP-cyclohydrolase I (GTP-CH, a rate-controlling enzyme in de novo synthesis of BH4) activity using radiochemical and chromatographic methods. Marked changes in NO synthesis, cNOS and iNOS activities, GTP-CH activity, and concentrations of BH4 and NADPH occurred in all placental and endometrial tissues between Days 30 and 140 of gestation. NO synthesis peaked on Day 60 of gestation in both intercotyledonary placenta and placentomes and on Days 40–60 in intercaruncular endometrium. NO synthesis in placentomes increased 100% between Days 80 and 100 of gestation, when placental and uterine blood flows increase continuously. In all placental and endometrial tissues, NO synthesis was positively correlated with total NOS activity, GTP-CH activity, and concentrations of BH4 and NADPH. Importantly, these results indicate a high degree of metabolic coordination among the several integrated pathways that support high rates of NO synthesis in the conceptus and uterus and establish a new base of information for future studies to define the roles of NO in fetal-placental growth and development.**

*conceptus, nitric oxide, placenta, pregnancy, uterus*

## INTRODUCTION

The placentae of all mammalian species undergo rapid formation of new blood vessels (angiogenesis) and marked growth during pregnancy [1, 2]. Placental angiogenesis is necessary to increase placental-fetal blood flow and the transfer of nutrients from maternal to fetal blood. Therefore, placental growth is crucial for controlling the survival, growth, and development of the fetus, and a better understanding of factors that regulate placental growth is essential to improving reproductive efficiency of domestic animals and humans. The sheep has a synepitheliochorial placenta, whose growth is maximal between Days 20 and 60 of gestation (term is 147 days) [3, 4]. The ovine placenta has 60–100 individual cotyledons formed by the attachment of fetal trophoblast cells at predetermined sites (caruncles) in the uterine endometrium, as well as the intercotyledonary chorioallantoic placenta [3].

Nitric oxide (NO), which is synthesized from L-arginine by NO synthases (NOS), is a key regulator of angiogenesis, early mammalian embryogenesis, placental trophoblast growth, and conceptus development in the uterus [5]. There are three isoforms of the NOS: neuronal NOS (nNOS or type I), inducible NOS (iNOS or type II), and endothelial NOS (eNOS or type III), all of which require tetrahydrobiopterin (BH4) and NADPH as essential cofactors [6]. In most tissues, nNOS and eNOS are constitutively expressed, and are collectively termed constitutive NOS (cNOS), whereas iNOS is induced by inflammatory cytokines and hormones. Constitutive NOS, but not iNOS, requires $Ca^{2+}$ for enzymatic activity. Both cNOS and iNOS are expressed in placentae of mammals, including humans, pigs, and sheep [7–10]. In ovine placenta, cNOS is composed primarily of eNOS but little nNOS [9]. Although there are reports of NOS expression and NO synthesis in ovine placenta during late gestation (Days 110–142) [11], little is known about changes in NO synthesis, NOS activity, or its cofactors in ovine utero-placental tissues associated with conceptus development.

We recently reported marked increases in concentrations of both arginine and its precursor citrulline in ovine allantoic fluids (a reservoir of nutrients for the fetal-placental tissues) between Days 30 and 60 of gestation [12]. Interestingly, these temporal changes coincide with the period of most rapid growth of the ovine placenta [3, 4]. On the basis of this observation, we hypothesized that placental NO synthesis was maximal during the first half of pregnancy. This hypothesis was tested in ewes between Day 30 and Day 140 of gestation by analyzing both placental and endometrial tissues because uterine functions are closely associated with placental development and function [13].

## MATERIALS AND METHODS

### *Chemicals*

BH4, HEPES, $N^G$-monomethyl-L-arginine, biopterin, NADPH, GTP, arginine, dithiothreitol (DTT), EGTA, EDTA, aprotinin, chymostatin, phenylmethylsulfonylfluoride, pepstatin, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and calmodulin were purchased from Sigma Chemicals (St. Louis, MO). L-[U-$^{14}$C]arginine was obtained

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from American Radiolabeled Chemicals (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade water and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Nitrate reductase and alkaline phosphatase were procured from Roche (Indianapolis, IN). Dowex 50W-X8 resin ($H^+$ form, 200–400 mesh) was purchased from Bio-Rad (Richmond, CA) and was converted to the $Na^+$ form before use as recommended by the manufacturer.

## Experimental Animals

Columbia crossbred ewes were mated to Suffolk rams when detected in estrus (Day 0) and 12 and 24 h later. Ewes were then assigned randomly to be hysterectomized (n = 4 per day) on Day 30, 40, 60, 80, 100, 120, or 140 of gestation to allow collection of placental and endometrial tissues. Because there are marked changes in physiological parameters in the ovine conceptus during pregnancy [12, 14] and because coefficients of variations for all measured parameters were relatively small (<5%–8%), we used four ewes per day of gestation on the basis of statistical power calculations. In preliminary studies, we determined that rates of NO synthesis in placental and endometrial tissues were not affected by the number of fetuses (data not shown). Thus, samples were obtained from one randomly selected fetus per ewe for this study when there were twin fetal lambs. None of the ewes in the study had more than two fetuses. Throughout gestation, ewes had free access to water and were fed individually 1.4 kg/day of an alfalfa-based diet containing 90.9% dry matter, 58.7% total digestible nutrients, 15.8% crude protein, 3.7% fat, 27.0% acid detergent fiber, 35.0% neutral detergent fiber, 0.065% vitamin mixture, and 0.15% salt mixture that met National Research Council (NRC) requirements [12]. Ewes consumed all of the feed provided daily. This study was approved by the Texas A&M University Institutional Agricultural Animal Care and Use Committee.

## Hysterectomy and Sample Collection

Hysterectomies were performed between 0800 and 0900 h, 24 h after the last feeding [12]. All ewes were administered isofluorane (5%) via an inhalation mask to induce anesthesia, which was maintained with isofluorane (1%–5%). A midventral laparotomy was performed to expose the reproductive tract. Placentomes, intercotyledonary placenta, and intercaruncular endometrium were obtained on all indicated days of gestation. A portion of these tissues was used immediately for metabolic studies and BH4 analysis, and the remaining tissues were stored at −80°C for enzyme assays and NADPH analysis within 1 wk. In this study, we did not separate the placentomes into maternal and fetal components because such a procedure would require a prolonged period of time to complete, which might compromise the biochemical viability of the tissues for metabolic studies.

## Determination of NO Synthesis

Placental and endometrial tissues (~200 mg) were rinsed three times with 1 ml of oxygenated (95% $O_2$:5% $CO_2$; v/v) basal medium Eagle containing 0.4 mM L-arginine, 0.5 mM L-glutamine, 5 mM D-glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B, preincubated at 37°C for 0.5 h in 4 ml of oxygenated basal medium Eagle and then incubated at 37°C for 6 h in 0.5 ml fresh oxygenated basal medium Eagle. Concentrations of other amino acids in the basal medium Eagle were as follows (mM): alanine, 0.4; asparagine, 0.05; aspartate, 0.05; glutamate, 0.1; cystine, 0.05; glycine, 0.3; histidine, 0.05; isoleucine, 0.2; leucine, 0.2; lysine, 0.2; methionine, 0.1; phenylalanine, 0.1; proline, 0.2; serine, 0.2; threonine, 0.2; tryptophan, 0.1; tyrosine, 0.1; and valine, 0.2. At the end of a 6-h incubation period, media were analyzed for nitrite plus nitrate (stable oxidation products of NO). In all experiments, medium incubated without cells was run as the blank.

Nitrite and nitrate in culture medium were analyzed by HPLC as previously described [15]. Briefly, 100 μl medium (diluted 1:2) was incubated for 10 min at room temperature with 10 μl of 316 μM 2,3-diaminonaphthalene, followed by addition of 5 μl of 2.8 M NaOH. The derivative was separated on a $C_8$ column (150 × 4.6 mm inner diameter) using 15 mM sodium phosphate buffer (pH 7.5; 50% methanol; flow rate, 1.3 ml/min) and detected with excitation at 375 nm and emission at 415 nm. Nitrate in culture medium was measured using this HPLC method after its conversion to nitrite by nitrate reductase [15]. Nitrite and nitrate were quantified using $NaNO_2$ and $NaNO_3$ standards, respectively.

## Determination of NOS Activity

The activities of total NOS, cNOS, and iNOS in placental and endometrial tissues were measured using [$^{14}$C]arginine [16]. Briefly, tissues (~300 mg) were homogenized in 1 ml of 50 mM buffer containing 1 mM DTT, 1 mM EDTA, and protease inhibitors (5 μg/ml phenylmethylsulfonyl-fluoride, 5 μg/ml aprotinin, 5 μg/ml chymostatin, and 5 μg/ml pepstatin). The homogenates were centrifuged at 600 × *g* for 15 min, and the supernatants were used for NOS assays. Total NOS activity was determined by mixing 100 μl of tissue extract with 50 μl of 8 mM $CaCl_2$, and 50 μl of reagent mixture (4 mM DTT, 4 mM $MgCl_2$, 0.4 mM [U-$^{14}$C]arginine [5 × $10^3$ dpm/nmol], 0.4 mM citrulline, 20 mM valine [an inhibitor of arginase], 0.4 mM NADPH, 0.4 mM BH4, 0.4 mM FAD, 0.4 mM FMN, 10 μg calmodulin, and 0.4 M HEPES, pH 7.4). The iNOS activity was measured by mixing 100 μl of tissue extract with 50 μl of the reagent mixture, 25 μl of 16 mM EGTA, and 25 μl $H_2O$. A blank was prepared by mixing 100 μl of tissue extract with 50 μl of the reagent mixture, 25 μl of 16 mM EGTA, and 25 μl of 16 mM $N^G$-monomethyl-L-arginine (an inhibitor of NOS). All assay tubes were incubated at 37°C for 30 min. Reactions were terminated by addition of 50 μl of 1.5 M $HClO_4$. Neutralized solution (0.5 ml) was loaded into an AG 50W-X8 resin ($Na^+$ form) column (0.55 × 6 cm) and the column eluted with 4 ml $H_2O$. The eluate containing [$^{14}$C]citrulline was measured for radioactivity. NOS activity was calculated on the basis of [$^{14}$C]citrulline production and medium specific activity (SA) of [$^{14}$C]arginine. Total NOS activity = ($^{14}$C dpm in the total NOS tube − $^{14}$C dpm in the blank tube)/[$^{14}$C]arginine SA. The iNOS activity = ($^{14}$C dpm in the iNOS tube − $^{14}$C dpm in the blank tube)/[$^{14}$C]arginine SA. The cNOS activity = total NOS activity − iNOS activity.

## Determination of GTP Cyclohydrolase I Activity

GTP cyclohydrolase I (GTP-CH) activity in placental and endometrial tissues was determined using HPLC [16]. Briefly, placental tissues (~300 mg) were homogenized in 1 ml of 100 μM phenylmethylsulfonyl fluoride and 0.1 M Tris buffer (pH 7.8, 0.3 M KCl, 2.5 mM EDTA, 10% glycerol). The tissue homogenate was centrifuged at 600 × *g* for 15 min. The supernatant fluid was loaded on a Sephadex G-25 column (5 × 60 mm; Amersham Biosciences, Little Chalfont, UK), followed by washing with 0.45 ml of 0.1 M Tris buffer. An additional 0.5 ml of 0.1 M Tris buffer was added to the column and the eluate collected for enzyme assays. The desalting of tissue extracts removed small molecules (e.g., amino acids and biopterin) that potentially interfere with GTP-CH analysis. An aliquot (200 μl) of the desalted enzyme preparation was mixed with 100 μl of 6 mM GTP in a brown microcentrifuge tube and the solution was incubated at 37°C in the dark. After 90 min, 25 μl of 1% $I_2$/2% KI (in 1 M HCl) was added to the tube. A separate blank consisted of 200 μl of the desalted enzyme preparation and 25 μl of 1% $I_2$/2% KI (in 1 M HCl); after 5 min, 100 μl of 6 mM GTP was added to the blank tube. All tubes were then centrifuged at 10 000 × *g* for 1 min. The supernatant fluid was mixed with 25 μl of 114 mM ascorbic acid, followed by neutralization with 25 μl of 1 M NaOH. Alkaline phosphatase was added to each tube (10 U/tube). After a 60-min incubation at 37°C in the dark, 50 μl of the solution was analyzed for neopterin on a Phenosphere 5 ODS-1 column (4.6 mm × 25 cm, 5 μm; Phenomenex, Torrance, CA) using isocratic elution (flow rate of 1 ml/min) and fluorescence detection (excitation 350 nm and emission 440 nm). The mobile phase solvent was 5% HPLC-grade methanol, 95% HPLC-grade water, and 7.5 mM sodium phosphate (pH 6.35).

## Determination of BH4

BH4 was analyzed by HPLC as described by Meininger and Wu [16]. Briefly, tissues (~50 mg) were homogenized in 0.5 ml of 0.1 M phosphoric acid containing 5 mM dithioerythritol and 60 μl of 2 M trichloacetic acid (TCA). BH4 standard (50 pmol/ml) or cell extract (100 μl) was mixed with 15 μl of 0.2 M TCA and 15 μl of acidic oxidizer (1% $I_2$/2% KI in 0.2 M TCA) (acidic oxidation) or with 15 μl of 1 M NaOH and 15 μl of alkaline oxidizer (1% $I_2$/2% KI in 3 M NaOH) (alkaline oxidation). After a 1-h incubation at 25°C in the dark, excess iodine was removed by adding 25 μl of 114 mM ascorbic acid. After neutralization, 50 μl of the solution was analyzed for biopterin as described above for neopterin. The amount of BH4 in tissue extracts was determined by subtracting the amount of biopterin measured after alkaline oxidation from the amount of biopterin measured after acidic oxidation.

## Determination of NADPH

NADPH in placental and endometrial tissues was determined using HPLC as described previously [17]. Briefly, 100 mg tissue was homogenized in 1 ml of 1 mM bathophenanthrolinedisulfonic acid/250 mM KOH. Ice-cold 1 M $KH_2PO_4$ (250 μl) and 1 ml of 1 mM bathophenanthroline-

TABLE 1. Nitric oxide synthase activities (nmol g tissue$^{-1}$ h$^{-1}$) in ovine placenta and endometrium.*

| | Day of gestation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 30 | 40 | 60 | 80 | 100 | 120 | 140 | SEM |
| Intercotyledonary placenta | | | | | | | | |
| Inducible NOS | 0.18$^{d}$ | 0.51$^{c}$ | 1.52$^{a}$ | 0.54$^{c}$ | 1.09$^{b}$ | 0.56$^{c}$ | 0.42$^{c}$ | 0.08 |
| Constitutive NOS | 0.04$^{d}$ | 0.44$^{bc}$ | 0.49$^{bc}$ | 1.03$^{a}$ | 0.36$^{c}$ | 0.43$^{bc}$ | 0.58$^{b}$ | 0.08 |
| Total NOS | 0.22$^{d}$ | 0.95$^{c}$ | 2.01$^{a}$ | 1.57$^{b}$ | 1.46$^{b}$ | 0.97$^{c}$ | 1.00$^{c}$ | 0.10 |
| Placentome | | | | | | | | |
| Inducible NOS | 1.44$^{e}$ | 2.01$^{d}$ | 3.13$^{c}$ | 2.24$^{d}$ | 6.02$^{b}$ | 8.45$^{a}$ | 5.68$^{b}$ | 0.24 |
| Constitutive NOS | 1.26$^{d}$ | 2.59$^{c}$ | 5.33$^{a}$ | 3.82$^{b}$ | 2.91$^{c}$ | 1.02$^{d}$ | 2.55$^{c}$ | 0.22 |
| Total NOS | 2.71$^{d}$ | 4.60$^{c}$ | 8.46$^{a}$ | 6.05$^{b}$ | 8.93$^{a}$ | 9.47$^{a}$ | 8.24$^{a}$ | 0.31 |
| Intercaruncular endometrium | | | | | | | | |
| Inducible NOS | 0.37$^{f}$ | 0.69$^{e}$ | 3.26$^{a}$ | 1.80$^{cd}$ | 1.47$^{d}$ | 2.37$^{b}$ | 2.12$^{bc}$ | 0.20 |
| Constitutive NOS | 0.42$^{f}$ | 7.60$^{a}$ | 5.69$^{b}$ | 1.91$^{e}$ | 1.80$^{e}$ | 3.44$^{c}$ | 2.41$^{d}$ | 0.39 |
| Total NOS | 0.79$^{e}$ | 8.28$^{a}$ | 8.95$^{a}$ | 3.70$^{d}$ | 3.26$^{d}$ | 5.81$^{b}$ | 4.53$^{c}$ | 0.37 |

* Data are the mean with pooled SEM values for four ewes per gestational age; means with different superscript letters within a row are different ($P < 0.01$).

disulfonic acid were added sequentially to the homogenates, followed by centrifugation (3000 × *g*, 15 min). An aliquot of the supernatant (25 μl) was analyzed on a Phenosphere 5 ODS-1 column (4.6 × 25 cm, 5 μm) using isocratic elution (1 ml/min) and fluorescence detection (excitation 340 nm, emission 460 nm). The mobile-phase solution was 150 mM potassium phosphate/5 mM tetrabutylammonium hydrogen sulfate/23% methanol (pH 7.5). NADPH in samples was quantified on the basis of NADPH standard.

### *Calculations and Statistical Analysis*

NOS and GTP-CH activities as well as BH4 and NADPH concentrations in placentae and endometria were calculated on the basis of tissue weight. Data were subjected to least squares analyses of variance, one-way analysis of variance, and correlation analysis [18], using the PROC GLM and PROC CORR procedures of SAS (SAS Institute, Inc., Cary, NC). Differences between means were determined by the Student-Newman-Keuls multiple comparison test following one-way analysis of variance [18]. Statistical significance was set at a probability value of ≤0.05.

## RESULTS

### *NOS Activity in Placental and Endometrial Tissues*

Marked changes in NOS activities occurred ($P < 0.01$) in ovine placental and endometrial tissues during conceptus development (Table 1). In intercotyledonary placenta, iNOS activity increased ($P < 0.01$) approximately 7.5-fold between Days 30 and 60 of gestation, declined ($P < 0.01$) on Day 80 of gestation, and increased again on Day 100 of gestation. The cNOS activity in this tissue increased ($P < 0.01$) 10-fold between Days 30 and 40 of gestation, further increased ($P < 0.01$) to Day 80 of gestation, and declined thereafter. Intercotyledonary placental cNOS activities were similar to ($P > 0.05$), greater than ($P < 0.01$), and lower than ($P < 0.01$) iNOS activities on Days 40, 120, and 140, Day 80, and Days 30, 60, and 100 of gestation, respectively. In intercaruncular endometrium, iNOS activity increased ($P < 0.01$) approximately 8-fold between Days 30 and 60 of gestation, declined ($P < 0.01$) by Days 80–100 of gestation, and increased again on Days 120–140 compared with Day 100 of gestation. Endometrial cNOS activity increased ($P < 0.01$) 17-fold between Days 30 and 40 of gestation, declined ($P < 0.01$) progressively between Days 40 and 80 of gestation, and increased again on Days 120–140 compared with Day 100 of gestation. In intercaruncular endometrium, cNOS activity was similar to ($P > 0.05$) and greater than ($P < 0.01$) iNOS activity on Days 30, 80, 100, and 140 and on Days 40, 60, and 120 of gestation, respectively. Placentomes exhibited the highest ($P < 0.01$) NOS activity among the placental and endometrial tissues during early (Day 30) and late (Days 80–140) gestation. Placentomal iNOS and cNOS activities increased ($P < 0.01$) progressively between Days 30 and 60 of gestation and then declined ($P < 0.01$) by Day 80 of gestation. In placentomes, iNOS activities increased ($P < 0.01$) but cNOS activities decreased ($P < 0.01$) between Days 80 and 120 of gestation, while total NOS activity remained constant. Placentomal cNOS activities were similar to ($P > 0.05$), greater than ($P < 0.01$), and lower than ($P < 0.01$) iNOS activities during early (Days 30–40), mid- (Days 60–80), and late (Days 100–140) gestation, respectively. Total NOS activity peaked ($P < 0.01$) on Days 40–60 of gestation in intercaruncular endometrium and on Day 60 of gestation in both intercotyledonary placenta and placentomes.

### *NO Synthesis in Placental and Endometrial Tissues*

These data are summarized in Table 2. In intercotyledonary placenta, NO production increased ($P < 0.01$) 160% between Days 30 and 60 of gestation and declined ($P < 0.01$) thereafter. In intercaruncular endometrium, NO synthesis increased ($P < 0.01$) 150%–170% on Days 40–60 compared with Day 30 of gestation and declined ($P < 0.01$) on Days 80–100 of gestation. Endometrial NO synthesis increased on Days 120–140 compared with Days 80–100 of gestation. Placentomes exhibited the highest ($P <$

TABLE 2. Nitric oxide synthesis (nmol g tissue$^{-1}$ h$^{-1}$) in ovine placenta and endometrium.*

| | Day of gestation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 30 | 40 | 60 | 80 | 100 | 120 | 140 | SEM |
| Intercotyledonary placenta | 0.25$^{d}$ | 0.47$^{b}$ | 0.64$^{a}$ | 0.49$^{b}$ | 0.47$^{b}$ | 0.38$^{c}$ | 0.36$^{c}$ | 0.02 |
| Placentome | 0.29$^{d}$ | 0.89$^{c}$ | 3.15$^{a}$ | 0.86$^{c}$ | 1.79$^{b}$ | 1.73$^{b}$ | 1.66$^{b}$ | 0.10 |
| Intercaruncular endometrium | 0.26$^{d}$ | 0.66$^{a}$ | 0.70$^{a}$ | 0.35$^{c}$ | 0.33$^{c}$ | 0.47$^{b}$ | 0.45$^{b}$ | 0.03 |

* Data are the mean with pooled SEM values for four ewes per gestational age; means with different superscript letters within a row are different ($P < 0.01$).

TABLE 3. GTP cyclohydrolase I activity (nmol g tissue$^{-1}$ h$^{-1}$) in ovine placenta and endometrium.*

| | Day of gestation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 30 | 40 | 60 | 80 | 100 | 120 | 140 | SEM |
| Intercotyledonary placenta | 0.84$^{d}$ | 1.96$^{b}$ | 2.73$^{a}$ | 2.01$^{b}$ | 1.93$^{b}$ | 1.44$^{c}$ | 1.36$^{c}$ | 0.11 |
| Placentome | 1.90$^{d}$ | 5.38$^{c}$ | 15.3$^{a}$ | 5.17$^{c}$ | 10.8$^{b}$ | 10.4$^{b}$ | 10.2$^{b}$ | 0.53 |
| Intercaruncular endometrium | 0.89$^{d}$ | 3.07$^{a}$ | 3.22$^{a}$ | 1.63$^{c}$ | 1.75$^{c}$ | 2.37$^{b}$ | 2.34$^{b}$ | 0.14 |

* Data are the mean with pooled SEM values for four ewes per gestational age; means with different superscript letters within a row are different ($P < 0.01$).

0.01) rate of NO synthesis among the placental and endometrial tissues between Days 30 and 140 of gestation. Placentomal NO production increased ($P < 0.01$) 10-fold between Days 30 and 60 of gestation, declined ($P < 0.01$) on Day 80 of gestation, and increased ($P < 0.01$) again during late gestation (Days 100–140) compared with Day 80 of gestation. In all placental and endometrial tissues, NO synthesis did not differ ($P > 0.05$) between Days 120 and 140 of gestation. Rates of NO generation peaked ($P < 0.01$) on Days 40–60 of gestation in intercaruncular endometrium and on Day 60 of gestation in both intercotyledonary placenta and placentomes.

### *GTP-CH Activity in Placental and Endometrial Tissues*

GTP-CH activity was the highest ($P < 0.01$) in placentomes among the ovine placental and endometrial tissues studied between Days 30 and 140 of gestation (Table 3). Throughout pregnancy, marked changes in GTP-CH activity occurred ($P < 0.01$) in placentomes and, to a lesser extent, in intercotyledonary placenta and intercaruncular endometrium. In intercotyledonary placenta, GTP-CH activity increased ($P < 0.01$) 225% between Days 30 and 60 of gestation and declined ($P < 0.01$) thereafter. In intercaruncular endometrium, GTP-CH activity increased ($P < 0.01$) 245%–260% on Days 40–60 compared with Day 30 of gestation, and then declined ($P < 0.01$) on Days 80–100 of gestation. Endometrial GTP activity increased ($P < 0.01$) approximately 35% on Days 120–140 compared with Days 80–100 of gestation. Strikingly, placentomal GTP activity increased ($P < 0.01$) 7-fold between Days 30 and 60 of gestation, declined ($P < 0.01$) markedly on Day 80 of gestation, and increased by approximately 100% ($P < 0.01$) on Days 100–140 compared with Day 80 of gestation. In all placental and endometrial tissues, GTP activity did not differ ($P > 0.05$) between Days 120 and 140 of gestation. GTP activity peaked ($P < 0.01$) on Days 40–60 of gestation in intercaruncular endometrium and on Day 60 of gestation for both intercotyledonary placenta and placentomes.

### *BH4 and NADPH Concentrations in Placental and Endometrial Tissues*

These data are summarized in Table 4. Placentomes exhibited the highest levels of BH4 and the most dramatic changes ($P < 0.01$) during pregnancy. Interestingly, BH4 levels were similar ($P > 0.05$) between intercotyledonary placenta and intercaruncular endometrium throughout gestation. In intercotyledonary placenta, BH4 levels increased ($P < 0.01$) by 180% between Days 30 and 60 of gestation and declined ($P < 0.01$) thereafter. In intercaruncular endometrium, BH4 levels doubled ($P < 0.01$) between Day 30 and Days 40–60 of gestation and then declined ($P < 0.01$) approximately 37% by Days 80–100 of gestation. Endometrial BH4 levels increased ($P < 0.01$) by approximately 30% on Days 120–140 compared with Days 80–100 of gestation. Remarkably, placentomal BH4 concentrations increased ($P < 0.01$) 8.4-fold between Days 30 and 60 of gestation, declined ($P < 0.01$) to Day 80 of gestation, and increased 2-fold ($P < 0.01$) on Days 100–140 compared with Day 80 of gestation. In all placental and endometrial tissues, BH4 levels did not differ ($P > 0.05$) between Days 120 and 140 of gestation.

The patterns of change in NADPH levels during pregnancy were similar among intercotyledonary placenta, placentome, and intercaruncular endometrium (Table 4). In all of these tissues, NADPH levels increased ($P < 0.01$) progressively between Days 30 and 60 of gestation, declined ($P < 0.01$) by Day 80 of gestation, and did not differ ($P > 0.05$) between Days 80 and 140 of gestation. Placentomes exhibited the highest ($P < 0.01$) levels of NADPH among the placental and endometrial tissues examined between Days 30 and 140 of pregnancy.

### *Correlations Between NO Synthesis and NOS Activity, GTP-CH Activity, and NOS Cofactors*

Rates of NO synthesis were positively correlated ($P < 0.01$) with NOS activity, GTP-CH activity, BH4 levels, and

TABLE 4. Tetrahydrobiopterin and NADPH concentrations (nmol/g tissue) in ovine placenta and endometrium.*

| | Day of gestation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 30 | 40 | 60 | 80 | 100 | 120 | 140 | SEM |
| Intercotyledonary placenta | | | | | | | | |
| Tetrahydrobiopterin | 0.24$^{d}$ | 0.52$^{b}$ | 0.68$^{a}$ | 0.55$^{b}$ | 0.53$^{b}$ | 0.40$^{c}$ | 0.38$^{c}$ | 0.02 |
| NADPH | 3.19$^{c}$ | 5.63$^{b}$ | 8.10$^{a}$ | 6.24$^{b}$ | 5.97$^{b}$ | 6.02$^{b}$ | 5.74$^{b}$ | 0.23 |
| Placentome | | | | | | | | |
| Tetrahydrobiopterin | 0.43$^{d}$ | 1.26$^{c}$ | 4.04$^{a}$ | 1.12$^{c}$ | 2.26$^{b}$ | 2.43$^{b}$ | 2.11$^{b}$ | 0.09 |
| NADPH | 4.20$^{c}$ | 9.11$^{b}$ | 12.3$^{a}$ | 9.06$^{b}$ | 8.43$^{b}$ | 8.61$^{b}$ | 8.32$^{b}$ | 0.42 |
| Intercaruncular endometrium | | | | | | | | |
| Tetrahydrobiopterin | 0.26$^{d}$ | 0.75$^{a}$ | 0.77$^{a}$ | 0.47$^{b}$ | 0.49$^{b}$ | 0.61$^{c}$ | 0.63$^{c}$ | 0.02 |
| NADPH | 3.45$^{c}$ | 5.98$^{b}$ | 8.72$^{a}$ | 6.51$^{b}$ | 6.23$^{b}$ | 6.01$^{b}$ | 6.14$^{b}$ | 0.18 |

* Data are the mean with pooled SEM values for four ewes per gestational age; means with different superscript letters within a row are different ($P < 0.01$).

NADPH levels in intercotyledonary placenta, placentomes, and intercaruncular endometrium (Table 5). Correlation coefficients between GTP-CH activity and BH4 levels were 0.89, 0.91, and 0.85 (n = 28; $P < 0.01$) in the same tissues, respectively.

TABLE 5. Correlations between NO synthesis and NOS activity, GTP-CH activity, BH4 level, and NADPH level in ovine placenta and endometrium.[a]

| | Total NOS activity | GTP-CH activity | BH4 level | NADPH level |
|---|---|---|---|---|
| Intercotyledonary placenta | 0.79 | 0.85 | 0.87 | 0.84 |
| Placentome | 0.75 | 0.94 | 0.95 | 0.72 |
| Intercaruncular endometrium | 0.87 | 0.92 | 0.83 | 0.67 |

[a] Values are Pearson correlation coefficients, n = 28. $P < 0.01$ for all values.

## DISCUSSION

Since the 1988 discovery of NO synthesis by macrophages and endothelial cells, there have been extensive studies of the role of NO in placental and fetal development [5, 19, 20]. The available evidence suggests that uterine synthesis of NO increases during pregnancy and decreases at the onset of parturition [5]. In addition, recent studies with the ovine model demonstrated that NO is a major mediator of placental-fetal blood flow during pregnancy [21]. Despite the report of NOS expression and NO synthesis in ovine placenta during late gestation (Days 110–142) [11], little is known about changes in NO production by ovine utero-placental tissues associated with conceptus development. Such information is crucial for understanding the molecular regulation of placental and fetal growth and for elucidating mechanisms responsible for intrauterine growth retardation and fetal origin of adult-onset diseases [22] that are related to disturbed NO metabolism [23, 24]. Because changes in NOS activity may not necessarily indicate changes in NO production by intact cells [6, 25], it is important that rates of NO synthesis be determined in placental and endometrial tissues to better understand the role of this pathway in conceptus development.

To our knowledge, this is the first report of NO synthesis, NOS and GTP-CH activities, as well as concentrations of BH4 and NADPH in placental and endometrial tissues of any species during early, mid-, and late pregnancy. There are four major findings from this study: 1) maximal activities of NOS and GTP-CH, the greatest availability of BH4 and NADPH, and the highest rate of NO synthesis occurred in ovine placenta and endometrium in the first half of pregnancy; 2) all of these measured parameters, except for NADPH, exhibited a second peak during late gestation when there is a continued increase in fetal-placental blood flows; 3) cNOS and iNOS activities varied greatly among ovine tissues and with gestational age; and 4) metabolic coordination occurred among the several integrated pathways that support high rates of NO synthesis in the placenta and endometrium.

The NOS (a dimer in its active form) is a flavoheme enzyme that contains binding sites for NADPH, FAD, and FMN in its reductase domain as well as arginine, BH4, and iron protoporphyrin IX (heme) in its oxygenase domain [6]. The C-terminal reductase domain is linked to the N-terminal oxygenase domain by a calmodulin-recognition site, which regulates electron transfer. In iNOS, $Ca^{2+}$ is tightly bound to calmodulin, and thus exogenous $Ca^{2+}$ is not required for its enzymatic activity. Although the precise role of BH4 in the NOS reaction is not fully understood, suggested functional roles include a) promotion of dimer formation, arginine binding, and NADPH oxidation; b) prevention of enzyme autoinactivation; and c) redox function [6]. Recent evidence shows that BH4 primarily plays a redox role in NOS catalysis, in which BH4 donates an electron to the heme of the oxygenase domain to form a BH4 radical ($BH4^{\bullet+}$), which then returns to the reduced state (BH4) by accepting an electron from a flavin in the reductase domain [26]. In the presence of $O_2$, NADPH, FAD, FMN, and BH4, all isoforms of the NOS catalyze oxidation of L-arginine to NO and L-citrulline, with $N^{\omega}$-hydroxyl-L-arginine as an enzyme-bound intermediate. L-Citrulline can be recyled into L-arginine via argininosuccinate synthase and lyase in virtually all animal cells [25]. For example, in ovine placentomes, argininosuccinate synthase and lyase activities were 0.85 ± 0.07 and 0.94 ± 0.11 nmol/mg protein/min (means ± SEM, n = 4), respectively, at Day 40 of gestation and increased ($P < 0.01$) to 1.73 ± 0.16 and 2.46 ± 0.21 nmol/mg protein/min (means ± SEM, n = 4) at Day 60 of gestation (unpublished results). The increase in enzyme activities is associated with increased arginine concentration in ovine allantoic fluid between Days 40 and 60 of gestation [12].

Because of the intracellular compartmentalization of NO synthesis and its functional consequence [25], much attention has been directed toward determining NOS isoforms in the female reproductive organs [e.g., 5, 7–10]. Both cNOS and iNOS are present in the gravid rat and ovine uterine tissues and may vary with species and gestational age [27–31]. For example, nNOS and eNOS were more predominant than iNOS in the ovine uterus during the last third of gestation [30], whereas the opposite was reported for the rat uterus throughout pregnancy [31]. In contrast, cNOS and iNOS activities were similar in both porcine placenta and endometrium during early and mid gestation [32]. Despite these reports, little is known about developmental changes in cNOS and iNOS activities in both placental and endometrial tissues throughout pregnancy. An interesting finding of this study was that cNOS and iNOS activities vary greatly with ovine placental and endometrial tissues and gestational age. For example, in placentomes, cNOS and iNOS activities predominated during mid- (Days 60–80) and late (Days 120–140) gestation, respectively. In intercaruncular endometrium, cNOS activity was 10-fold greater than, and similar to, iNOS activity on Days 40 and 140 of gestation, respectively. In contrast, in intercotyledonary placenta, cNOS activity was less and greater than iNOS activity on Days 30 and 80 of gestation, respectively. The changes in placental and endometrial NOS expression during gestation may be brought about by changes in many factors, including hormones [10, 20], cytokines [6], and uterine secretions [5], as well as nutrients and their metabolites [33].

Placental synthesis of NO, like that of polyamines (other products of arginine catabolism) essential for placental angiogenesis and growth) [14], increased markedly between Days 30 and 60 of gestation (Table 2) when placental growth and placentomal development are most rapid [3, 4]. Likewise, NO synthesis in intercaruncular endometrium peaked on Days 40–60 of gestation, when this tissue undergoes marked morphological and functional changes [13]. Results of the present study support our hypothesis that NO and polyamines are crucial for placental and endometrial growth during early pregnancy [5, 14] and, therefore, for

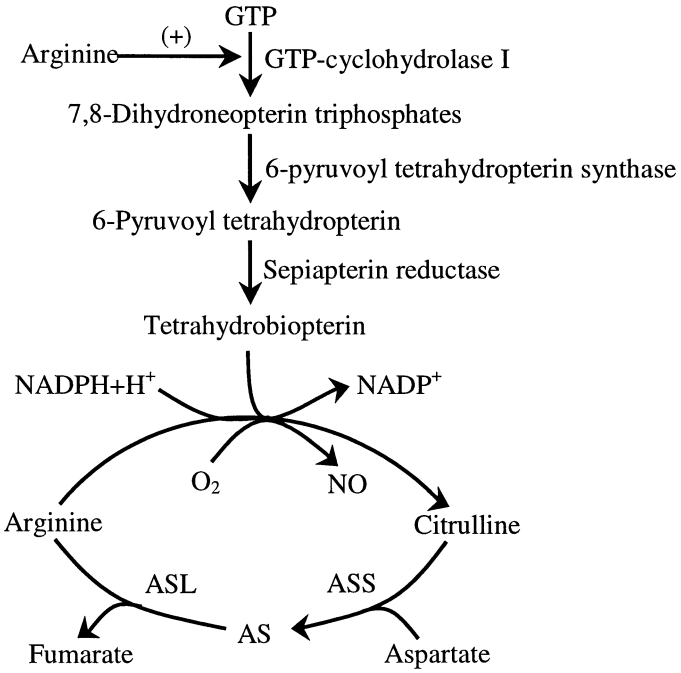


FIG. 1. Role of arginine, tetrahydrobiopterin, and NADPH in nitric oxide synthesis. AS, Argininosuccinate; ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; NO, nitric oxide. Tetrahydrobiopterin (BH4) primarily plays a redox role in NOS catalysis, in which BH4 donates an electron to the heme to form a BH4 radical ($BH4^{\bullet+}$), which then returns to the reduced state (BH4) by accepting an electron from a flavin.

fetal growth and development. In support of this view, inhibition of NOS or ornithine decarboxylase (a key enzyme in polyamine synthesis) activity during early pregnancy markedly reduced placental size and caused intrauterine growth retardation in rats [34–37]. It is noteworthy that there was a second peak in placentomal NO synthesis on Day 100 of gestation, when placental-fetal blood flow continues to increase in pregnant ewes [2, 4]. The increase in NO synthesis during late gestation may play an important role in enhancing the transfer of nutrients and oxygen from maternal to fetal blood to support the most rapid absolute growth of the fetus. For example, fetal weight gain between Days 120 and 140 of gestation is similar to that during the first 4 mo of gestation in sheep [12]. This necessitates an increased provision of nutrients (e.g., amino acids) for metabolic utilization (e.g., tissue protein synthesis and gluconeogenesis).

In the present study, it was not possible to discern whether the placentomal changes in NO synthesis were of placental or maternal origin because placentomes were not separated into cotyledonary and caruncular components. In ewes, vascular density of the cotyledonary bed remains relatively constant between Days 40 and 80 of gestation and then increases exponentially thereafter [38, 39]. In contrast, vascular density of caruncular tissues increases substantially until midgestation and then more slowly thereafter [39]. These data are consistent with the finding that umbilical blood flow increases more rapidly than uterine blood flow during the last half of gestation in ewes [40, 41]. On the basis of these observations, we surmise that the placentomal increase in NO synthesis between Days 30 and 60 and between Days 80 and 140 of gestation occurred primarily in caruncular tissues and the cotyledonary bed, respectively. Future studies are necessary to evaluate NO synthesis patterns in the fetal and maternal placentomal tissues to determine if the changes are related to the known differences in caruncular and cotyledonary angiogenesis and vascular development.

Although NADPH and BH4 are known to be essential cofactors for NO synthesis in cells and tissues [6], including human placenta [42], little is known about their concentrations in placenta and endometrium of any species during pregnancy. In animal cells, NADPH is produced primarily from glucose metabolism via the pentose cycle [43], whereas GTP-CH catalyzes the first and rate-controlling step in the de novo synthesis of BH4 from GTP [44]. Consistent with this view, changes in GTP-CH activity were positively correlated with changes in BH4 levels in ovine intercotyledonary placenta, placentome, and intercaruncular endometrium between Days 30 and 140 of gestation. Intriguingly, in ovine placenta and endometrium, both NADPH and BH4 levels increased markedly between Days 40 and 60 of gestation (Table 4), as did allantoic fluid concentrations of citrulline (the precursor of arginine) and arginine [12]. Between Days 80 and 100 of gestation, BH4 concentrations also increased in placentome and endometrium (Table 4), as did concentrations of arginine in allantoic fluid [12]. This amino acid, which is a potential regulator of the pentose cycle activity [43] and a stimulator of endothelial GTP-CH expression [45], may play an important role in regulating the synthesis of NADPH and BH4 and, therefore, NO production in placenta and endometrium. In support of this view, rates of NO synthesis in ovine placenta and endometrium were positively correlated with NADPH and BH4 levels during pregnancy (Table 5).

On the basis of water content of the ovine placentome (approximately 81%), intercotyledonary placenta (approximately 87%) and intercaruncular endometrium (approximately 74%) [14], mean concentrations of NADPH in these tissues were estimated to be 3.7–9.3 μM, 5.2–15.2 μM, and 4.7–11.2 μM, respectively. Mean concentrations of BH4 in the same tissues were estimated to be 0.28–0.78 μM, 0.53–5.0 μM, and 0.35–1.04 μM, respectively. Although these values were higher than the Michaelis constant (Km) of purified eNOS and iNOS for NADPH (~1 μM) and BH4 (~0.1 μM) [6], changes in tissue NADPH and BH4 levels can modulate both constitutive and inducible NO production [17, 46–48]. For example, increasing NADPH levels from 230 to 330 μM in endothelial cells through the metabolic stimulation of the pentose cycle increased NO synthesis by 62% [17]. Furthermore, increasing BH4 levels 140% through GTP-CH gene transfer increased NO production by 260% in carotid arteries of hypertensive rats [46]. Similarly, intracellular concentrations of arginine (~1–2 mM) are much higher than the Km of purified eNOS and iNOS for arginine (~3–20 μM) [6], but increasing tissue arginine above 2 mM stimulates NO production by both endothelial cells and activated macrophages [33]. This so-called paradox of NO synthesis may be explained by the compartmentalization of BH4, NADPH, and arginine [25]; multiple competitive pathways for their utilization [48]; and protein-protein interaction in intact cells [6].

Our findings indicate that patterns of NO synthesis as well as the availability of cofactors of NOS vary greatly among ovine placental and endometrial tissues. Whatever the differences, the highest rates of NO synthesis in these tissues occur when their growth is the most rapid during early gestation [3, 4, 13, 49, 50]. Additionally, our results suggest metabolic coordination among the several integrated pathways that support high rates of NO in placental and endometrial tissues (Fig. 1). For example, concentrations of

citrulline (the effective precursor of arginine) in ovine allantoic fluid increase 34-fold between Days 30 and 60 of gestation [12], thus increasing the availability of arginine for metabolism in placental and endometrium [14]. In addition, placentomal GTP-CH activity peaked on Day 60 of gestation (Table 3), thus maximizing the de novo synthesis of BH4. Consequently, placentomal BH4 and NADPH concentrations were highest on Day 60 of gestation (Table 4). All of these changes contribute to maximal NO production during early pregnancy. Our findings raise important questions regarding the physiological significance of NO synthesis in fetal-placental nutrition and development. It is noteworthy that maternal undernutrition decreases concentrations of arginine in fetal plasma and allantoic fluid and impairs fetal growth [51, 52] and may also program permanent structural, metabolic, and functional alterations [22, 53]. Maternal undernutrition in sheep (50% of NRC nutrient requirements) from Day 28 to Day 78 of gestation decreased concentrations of arginine and biopterin (an indicator of BH4 availability) in fetal plasma and allantoic fluids by 20%–38% (unpublished results) and reduced fetal growth by 32% on Day 78 of gestation [54]. Because recent epidemiological studies in humans suggest that there are links between intrauterine growth retardation and development of chronic disease (e.g., diabetes, hypertension, and coronary heart disease) later in life [22, 53], placental synthesis of NO may have important implications for both intrauterine growth retardation and fetal origins of diseases in adults.

In conclusion, results of the present study indicate that NO synthesis was highest in both placentomes and endometrium of ewes on Day 60 of gestation when their growth and morphological changes are most rapid and when fetal-placental blood flow increases substantially. Relatively high rates of NO synthesis occurred in the second half of pregnancy in association with further increases in the placental vascular bed and uterine blood flow to support rapid fetal growth. Importantly, there is metabolic coordination among the several integrated pathways that support high rates of NO synthesis in the ovine conceptus. These results establish a new base of information for future studies to define the roles of NO in fetal-placental growth and development.

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