Maternal Dietary Protein Deficiency Decreases Nitric Oxide Synthase and Ornithine Decarboxylase Activities in Placenta and Endometrium of Pigs During Early Gestation[1,2]

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ABSTRACT Little is known about the mechanism responsible for retarded placental and fetal growth induced by maternal dietary protein malnutrition. On the basis of the recent finding that nitric oxide (NO) and polyamines (products of L-arginine) play an important role in embryonic and placental development, the present study was designed to determine whether protein deficiency decreases placental and endometrial activities of NO synthase (NOS) and ornithine decarboxylase (ODC) (the first and key regulatory enzyme in polyamine synthesis). Primiparous gilts selected genetically for low or high plasma total cholesterol concentrations (low line and high line, respectively) were mated and then fed 1.8 kg/d of isocaloric diets containing 13% or 0.5% crude protein. At d 40 or 60 of gestation, they were hysterectomized, and placenta and endometrium were obtained for incubations, NOS and ODC assays, and measurements of free amino acids and polyamines. Maternal dietary protein restriction decreased arginine and ornithine concentrations, constitutive and inducible NOS activities and NO production, as well as ODC activity and polyamine concentrations in placenta and endometrium of both lines of gilts. Placental NO synthase activity and NO generation were lower in high line gilts than in low line gilts. ODC activities and polyamine concentrations in placenta and endometrium were decreased at d 60 compared with d 40 of gestation. These changes in placental and endometrial synthesis of NO and polyamines during early gestation may be a mechanism responsible for reduced placental and fetal growth in protein-deficient gilts and for altered conceptus development in high line gilts. J. Nutr. 128: 2395–2402, 1998.

KEY WORDS: • *protein malnutrition* • *nitric oxide synthase* • *ornithine* • *decarboxylase* • *fetus* • *pig*

Evvard et al. (1914) demonstrated that maternal dietary protein deficiency resulted in lower birth weights and decreased vigor of the offspring in pigs. Studies over the last three decades have established the stunting effect of protein restriction on intrauterine fetal growth in humans, pigs and rats (Atinmo et al. 1974 and 1976, Desai et al. 1996, Pond 1973, Pond et al. 1968, 1969, 1991 and 1992, Schoknecht et al. 1994, Widdowson 1977, Zeman and Stanbrough 1969). Protein deficiency during the first trimester of pregnancy has a greater detrimental effect on fetal development than during late pregnancy (Pond 1973, Pond et al. 1968, 1969, 1991 and 1992). These results suggest that during the peri-implantation period (e.g., 13–20 d of gestation in the pig) and the period of placental development (e.g., 20–60 d of gestation in the pig), fetal growth is most vulnerable to maternal protein deficiency. Protein malnutrition during gestation results not only in decreased growth of fetal organs, but also in permanent alterations in their structure, metabolism and function (Desai et al. 1996, Ozanne et al. 1997). These findings have important implications to both postnatal development and fetal origins of diseases, because the rate of fetal growth and subsequent birth weight are major determinants of postnatal survival and growth (Reynolds and Redmer 1995) and because recent epidemiological studies in humans suggest that there are links between impaired growth during early prenatal life and development of chronic diseases such as diabetes, hypertension and coronary heart disease later in life (Barker et al. 1990 and 1993).

The mechanism responsible for impaired fetal growth in protein-deficient dams remains largely unknown. Maternal and fetal serum concentrations of insulin-like growth factor I and II, as well as maternal serum concentrations of placental lactogen, decrease in protein-deficient pregnant rats (Pilistine

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et al. 1984). Interestingly, dietary protein deficiency has no effect on maternal plasma concentrations of amino acids but results in decreased concentrations of most amino acids (including arginine and ornithine) in fetal plasma and allantoic fluid of pigs (Wu et al. 1998). Protein malnutrition also reduced the size of placenta (Malandro et al. 1996, Pilistine et al. 1984, Schoknecht et al. 1994), a critical organ for normal growth and development of the fetus (Reynolds and Redmer 1995). The endometrium (a mucosal layer underlying placenta) is also important for early embryonic development, implantation, placentation and successful placental and fetal development (Bazer 1992).

Ornithine decarboxylase, the first and key regulatory enzyme in the synthesis of polyamines from L-ornithine (and ultimately L-arginine), is essential to placental growth (Hoshiai et al. 1981) and early mammalian embryogenesis (Fozard et al. 1980). Nitric oxide (NO[5]), synthesized from L-arginine by NO synthase (NOS) (Knowles and Moncada 1994), plays an important role in regulating angiogenesis (Ziche et al. 1994) [an early critical event in placental growth during pregnancy (Reynolds et al. 1992)], placental-fetal blood flow and therefore nutrient supply from maternal to fetal blood (Sooranna et al. 1995). Both constitutive NOS (cNOS, Ca^{2+}-dependent) and inducible NOS (iNOS, Ca^{2+}-independent) were recently identified in placenta (Sladek et al. 1997) and endometrium (Telfer et al. 1997). We hypothesize that maternal dietary protein deficiency decreases NOS and ODC activities in placenta and endometrium during early pregnancy, thereby contributing to retarded placental and fetal development. This hypothesis was tested in the present study with lines of gilts selected to exhibit low and high serum cholesterol levels (Pond et al. 1997). Gilts were fed a protein-restricted diet for the first 40 or 60 d of gestation. Recent studies have indicated decreased reproductive function in gilts of the high cholesterol line compared with gilts of the low cholesterol line (Wise et al. 1993); therefore, this study determined whether reduced reproductive function in high line gilts was associated with altered NOS and ODC activities in placenta and endometrium. Days 40 and 60 of pregnancy were selected for sample collections on the basis of previous findings that *1)* events in early gestation (e.g., d 40) are critical to porcine fetal growth and development (Bazer 1992, Pond and Maner 1984), *2)* porcine placental development is maximal by d 60 of gestation (Knight et al. 1977), *3)* porcine placental and fetal growth retardation in maternal protein deficiency is manifested by d 60 of gestation (Pond et al. 1992, Schoknecht et al. 1994), and *4)* arginine and ornithine are most abundant in porcine allantoic fluid at d 40 and 60 of gestation (Wu et al. 1995 and 1996a).

MATERIALS AND METHODS

Chemicals. L-Amino acids, D-glucose, HEPES, calmodulin, N^G-methyl-L-arginine, N^G-nitro-L-arginine, NADPH, FAD, FMN, EDTA, EGTA, dithiothreitol, (6R)-5,6,7,8-tetrahydro-L-biopterin, phenylmethylsulfonylfluoride, aprotinin, chymostatin, pepstatin, putrescine, spermidine, spermine, cadaverine, hexanediamine, *o*-phthaldialdehyde and sodium dodecyl sulfate were purchased from Sigma Chemicals (St. Louis, MO). L-[U-^{14}C]arginine and L-[1-^{14}C]ornithine were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Dowex 50W-X8 resin (H^+ form, 200–400 mesh) was purchased from Bio-Rad (Richmond, CA) and was converted to Na^+ form immediately before use as recommended by the manufacturer.

[5] Abbreviations used: cNOS, constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; ODC, ornithine decarboxylase.

TABLE 1

Experimental design[1]

Diet	Low line gilts[2]		High line gilts[3]	
	d 40 of gestation	d 60 of gestation	d 40 of gestation	d 60 of gestation
	n			
0.5% protein	3	2	3	2
13% protein	3	3	3	3

[1] On the day of mating (d 0 of gestation), gilts within each genetic line were assigned randomly to the 0.5% or 13% protein diet and to the 40- or 60-d pregnancy group in a 2 × 2 × 2 factorial design.
[2] Genetically low plasma concentrations of total cholesterol.
[3] Genetically high plasma concentrations of total cholesterol.

Pigs. This study was approved by Texas A&M University's Institutional Animal Care and Use Committee and by Baylor College of Medicine's Institutional Animal Care and Use Committee. Primiparous gilts selected genetically for low or high plasma total cholesterol concentrations (low line and high line, respectively) (Pond et al. 1997) were housed at the Swine Center of Sam Houston State University. Before mating, gilts were fed a control diet containing 13% crude protein, as previously described (Wu et al. 1998). On the day of mating (d 0 of gestation) gilts within each genetic line were assigned randomly to the control or the protein-deficient diet and to the 40- or 60-d pregnancy group in a 2 × 2 × 2 factorial design (n = 2–3 gilts per cell) (**Table 1**). Throughout gestation control and protein-deficient gilts had free access to water and were fed once daily 1.8 kg of diets containing 13% or 0.5% crude protein (Wu et al. 1998). Gilts were hysterectomized at d 40 or 60 of gestation. Twenty hours before hysterectomy gilts were transported from Sam Houston State University to Texas A&M University (College Station, TX) where sample collections were performed as described below, between 0830 and 0900 h, 24 h after the last feeding.

Collection of placenta and endometrium. All gilts were hysterectomized as described previously (Wu et al. 1996a). Briefly, pigs were injected intramuscularly with Telazol (2.2 mg/kg body wt) to induce anesthesia, which was maintained with halothane (1–5%). A midventral laparotomy was performed and the reproductive tract was exposed. Placenta and endometrium were carefully isolated from the uterus of individual fetuses, as previously described (Knight et al. 1977, Tuo et al. 1996). Portions of placental and endometrial tissues were used immediately for in vitro incubations and ODC assays, and remaining samples were placed in liquid nitrogen, stored at −80°C and used for NOS assays and measurements of free amino acids and polyamine concentrations.

Incubation of placenta and endometrium for measuring L-citrulline synthesis from L-arginine. Nitric oxide synthase catalyzes stoichiometrically the synthesis of 1 mol of NO and 1 mol of L-citrulline from 1 mol of L-arginine (Knowles and Moncada 1994). In preliminary studies, we determined that it was difficult to measure formation of nitrite plus nitrate (stable oxidation products of NO) in incubated placental and endometrial tissues (for 3 h) because of a low rate of NO production and relatively high blank values of nitrite and nitrate, and that although arginase activity was present in porcine placenta and endometrium, activities of ornithine carbamoyltransferase and carbamoylphosphate synthase I (mitochondrial enzymes required for converting ornithine into citrulline) were absent from these tissues when the enzymes were assayed by established methods (Wu 1995). Because there was no conversion of arginine into citrulline via an alternative pathway (arginase and ornithine carbamoyltransferase) in porcine placenta and endometrium, a sensitive radiochemical technique was used to measure NO synthesis by measuring generation of L-[^{14}C]citrulline from L-[^{14}C]arginine (Bredt and Schmidt 1996).

Placental and endometrial tissues (~200 mg) were incubated (70 oscillations per min) at 37°C for 3 h in 2 mL of oxygenated (95% O_2/5% CO_2) Krebs-Henseleit bicarbonate buffer (119 mmol/L NaCl,

4.8 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4 and 25 mmol/L $NaHCO_3$) containing 20 mmol/L HEPES, 5 mmol/L D-glucose, 0.2 mmol/L L-[U-^{14}C]arginine (50 Bq/nmol) and 10 mmol/L L-valine (an inhibitor of arginase). Blanks contained all the above components (including tissues) plus 2 mmol/L N^G-nitro-L-arginine, an inhibitor of NOS but not arginase (Wu et al. 1996b). N^G-nitro-L-arginine, unlike N^G-methyl-arginine, does not inhibit arginine uptake by mammalian cells (Baydoun and Mann 1994, Bogle et al. 1995). Incubations were terminated by addition of 0.2 mL 1.5 mol/L $HClO_4$. The acidified medium plus tissue was homogenized in a glass homogenizer and was neutralized with 0.1 mL 2 mol/L K_2CO_3 followed by addition of 1 mL 20 mmol/L HEPES buffer (pH 5.5). A portion (1 mL) of neutralized samples was loaded into Dowex 50W-X8 resin (Na^+ form) (0.6 × 6.5 cm), and L-[^{14}C]citrulline was eluted from the column with 4 mL H_2O with recovery of 96% of a known amount of [^{14}C]citrulline standard. ^{14}C-Citrulline radioactivity was measured by a Packard 1900 liquid scintillation counter. Citrulline synthesis from arginine by NOS was calculated on the basis of medium specific radioactivity of L-[U-^{14}C]arginine and [^{14}C]citrulline production after correction for blank radioactivity values.

ODC assay. ODC activity was measured as previously described (Wu et al. 1996b). Placental and endometrial tissues (~0.5 g) were homogenized in 2 mL of a buffer containing 0.1 mmol/L pyridoxal-5-phosphate, 0.1 mmol/L EDTA, 2.5 mmol/L dithiothreitol, protease inhibitors (5 mg/L phenylmethylsulfonylfluoride, 5 mg/L aprotinin, 5 mg/L chymostatin and 5 mg/L pepstatin) and 50 mmol/L sodium phosphate buffer (pH 7.2). The homogenizer was rinsed with 1 mL of the buffer, and the combined homogenates were centrifuged at 13,000 × *g* and 4°C for 15 min. The supernatant fluid (free of mitochondria) was used for ODC assay. The ODC assay mixture (0.4 mL) consisted of 0.2 mmol/L L-[1-^{14}C]ornithine (100 Bq/nmol), 0.2 mmol/L pyridoxal-5-phosphate, 0.5 mmol/L dithiothreitol, 50 mmol/L sodium phosphate buffer (pH 7.2) and tissue cytosolic fractions (~1 mg protein). Radioactivity blanks containing all components except for tissue extracts were run along with the samples. After incubation for 1 h at 37°C, $^{14}CO_2$ was collected in 0.2 mL of NCS, and its radioactivity was measured by a Packard 1900 liquid scintillation counter.

NOS assay. The activities of total NOS, cNOS and iNOS were measured as described previously (Weiner et al. 1994, Wu et al. 1996b). Placental and endometrial tissues (~0.5 g) were homogenized in 1 mL 50 mmol/L HEPES buffer (pH 7.4) containing 1 mmol/L dithiothreitol, 1 mmol/L EDTA and protease inhibitors (5 mg/L phenylmethylsulfonyl-fluoride, 5 mg/L aprotinin, 5 mg/L chymostatin and 5 mg/L pepstatin). The homogenizer was rinsed with 1 mL of the buffer, and the combined homogenates were centrifuged at 600 × *g* and 4°C for 10 min. The supernatant fluid was used for NOS assay. For total NOS assay, the mixture (0.2 mL) contained 0.1 mmol/L (6R)-5,6,7,8-tetrahydro-L-biopterin, 1 mmol/L dithiothreitol, 1 mmol/L $MgCl_2$, 1 mg/L calmodulin, 0.1 mmol/L NADPH, 0.1 mmol/L FAD, 0.1 mmol/L FMN, 2 mmol/L $CaCl_2$, 0.1 mmol/L L-[U-^{14}C]arginine (150 Bq/nmol), 10 mmol/L L-valine, 0.1 mmol/L L-citrulline (to prevent recycling of [^{14}C]citrulline into arginine) and tissue extracts (~1 mg protein). For iNOS assay, the mixture contained all above components except $CaCl_2$, which was replaced with 2 mmol/L EGTA. Radioactivity blanks containing all above components plus 2 mmol/L N^G-methyl-L-arginine (an inhibitor of NOS) were analyzed to improve assay specificity. After incubation for 30 min at 37°C, reaction was terminated by addition of 100 μL 1.5 mol/L $HClO_4$. The acidified medium was neutralized with 40 μL 2 mol/L K_2CO_3 and then mixed with 1 mL 20 mmol/L HEPES (pH 5.5). A portion (0.5 mL) of neutralized samples was loaded into Dowex 50W-X8 resin (Na^+ form), and [^{14}C]citrulline was separated from [^{14}C]arginine as described above. cNOS activity was calculated by subtracting iNOS from total NOS activity (Weiner et al. 1994).

Determination of free amino acids and polyamines. Placental and endometrial tissues (~1 g) were homogenized at 4°C in 1 mL 1.5 mol/L $HClO_4$ using a glass homogenizer, and the homogenizer was rinsed with 1 mL 1.5 mol/L $HClO_4$. The combined acidified homogenates in 12 × 75 mm polypropylene tubes (which were used to avoid polyamine losses due to absorption to surfaces) were neutralized with 0.5 mL 2 mol/L K_2CO_3 and centrifuged at 3000 × *g* and 4°C for 15 min. Supernatant fluid was transferred to polypropylene tubes and analyzed for amino acids by an HPLC method as described previously (Wu et al. 1996a). Polyamines in the supernatant were determined by an ion-pairing reversed-phase HPLC method (Seiler and Knodgen 1985) with the following modifications: precolumn derivatization of amines with *o*-phthaldialdehyde, use of sodium dodecyl sulfate as an ion-pairing agent and solvent gradient. Our Waters HPLC apparatus consisted of a Model 600E Powerline multisolvent delivery system with 100 μL heads, a Model 712 WISP autosampler, a Supelco 3 μm reversed-phased C_{18} column (150 × 4.6 mm I.D.) guarded by a Supelco 4 μm reversed-phase C_{18} column (50 x 4.6 mm I.D.), a Model 420-AC fluorescence detector and a Model 810 Baseline Workstation (Waters Inc., Milford, MA). The mobile phase consisted of solvent A (0.1 mol/L sodium acetate, 2 mmol/L sodium dodecyl sulfate, 0.5% tetrahydrofuran and 9% methanol, pH 7.2) and solvent B (methanol and 2 mmol/L sodium dodecyl sulfate) with a combined total flow-rate of 1 mL/min. The assay mixture consisted of 200 μL sample, 200 μL 50 nmol/L hexanediamine (internal standard), 100 μL 1.2% benzoic acid (in 40 mmol/L sodium borate, pH 9.5) and 1.4 mL HPLC-grade water. An aliquot of the assay mixture (50 μL) was derivatized in Waters 712 WISP autosampler with 50 μL 30 mmol/L *o*-phthaldialdehyde (in 9.7% methanol, 3.1% Brij-35, 50 mmol/L 2-mercaptoethanol and 40 mmol/L sodium borate, pH 9.5). Fifty microliters of the derivatization mixture was injected into the column for separation. A gradient program with a total running time of 35 min was developed for satisfactory separation of polyamines, their N^1-acetyl derivatives and hexanediamine (0 min, 40% B; 3 min, 65% B; 10 min, 70% B; 27 min, 100% B; 28 min, 100% B; 28.1 min, 40% B; 35 min, 40% B). The retention times of N^1-acetylputrescine, N^1-acetylspermidine, putrescine, cadaverine, hexanediamine, spermidine, N^1-acetylspermine and spermine were 10.0, 17.0, 17.7, 19.8, 21.9, 22.7, 25.2 and 27.5 min, respectively. Polyamines were quantified on the basis of their standards using the Baseline Workstation.

Statistical analysis. Data were analyzed by three-way (2 × 2 × 2) factorial analysis of variance with the gilt as the experimental unit and fetuses nested within gilt (Steel and Torrie 1980). Statistical analysis was performed using general linear models (GLM) procedures of the SAS program (SAS 1990). Differences between means of main effects (diet, genetic line and gestational age) and their interactions were determined by the Student-Newman-Keuls multiple comparison test. Probability values < 0.05 were taken to indicate statistical significance.

RESULTS

Maternal dietary protein deficiency had profound effects on all measured parameters of this study [tissue concentrations of free basic amino acids (arginine, lysine and ornithine) and polyamines, NOS and ODC activities, and NO synthesis in placenta and endometrium] in low line and high line gilts. Placental and endometrial ODC activities, endometrial NOS activity and tissue concentrations of free amino acids and polyamines did not differ ($P > 0.05$) between low line and high line gilts. There were no significant interactions ($P > 0.05$) in any of these measurements among the diet, genetic line and gestational age.

Tissue concentrations of free amino acids. Concentrations of free arginine and ornithine in placenta and endometrium are summarized in **Table 2.** Placental and endometrial concentrations of other amino acids are shown in Appendix 1 and 2. Maternal dietary protein deficiency decreased ($P < 0.05$) concentrations of basic amino acids (arginine, lysine and ornithine) and several neutral amino acids (alanine, glutamine, glycine, branched-chain amino acids, proline, serine, taurine and threonine) in placenta and endometrium by 16–30%. There was no difference ($P > 0.05$) in placental or endometrial amino acid concentrations between low line and high line gilts. No difference was detected ($P > 0.05$) for amino acid concentrations in placenta or endometrium between d 40 and 60 of gestation.

TABLE 2

Concentrations of free arginine and ornithine in placenta and endometrium of gilts[1]

Amino acid	Dietary protein 0.5% (*n* = 10)	Dietary protein 13% (*n* = 12)	Gilts Low line[2] (*n* = 11)	Gilts High line[3] (*n* = 11)	Gestational age d 40 (*n* = 12)	Gestational age d 60 (*n* = 10)
			nmol/g wet tissue			
			Placenta			
Arg	202 ± 16	258 ± 7*	230 ± 12	236 ± 10	227 ± 11	240 ± 13
Orn	118 ± 6	170 ± 6*	150 ± 10	142 ± 10	145 ± 10	148 ± 11
			Endometrium			
Arg	180 ± 9	237 ± 6*	205 ± 13	208 ± 12	227 ± 11	240 ± 10
Orn	106 ± 6	142 ± 7*	127 ± 9	124 ± 10	122 ± 8	130 ± 9

* $P < 0.05$, different from the 0.5% protein group.
[1] Data are means ± SEM with the number of gilts in parentheses.
[2] Genetically low plasma concentrations of total cholesterol.
[3] Genetically high plasma concentrations of total cholesterol.

Nitric oxide synthase activities. **Table 3** summarizes activities of total NOS, iNOS and cNOS in placenta and endometrium. Maternal dietary protein deficiency decreased ($P < 0.05$) the activities of total NOS, iNOS and cNOS in placenta and endometrium by 30–51%. Activities of total NOS, iNOS and cNOS in placenta were lower ($P < 0.05$) in high line gilts than in low line gilts, but there were no differences ($P > 0.05$) in endometrial NOS activity between low line and high line gilts. Placental or endometrial activities of total NOS, iNOS or cNOS did not differ ($P > 0.05$) between d 40 and d 60 of gestation.

Citrulline synthesis from L-arginine by NO synthase. L-Citrulline production from L-arginine by NOS in incubated placenta and endometrium is shown in **Table 4.** Maternal dietary protein deficiency decreased ($P < 0.05$) citrulline synthesis from arginine in placenta and endometrium by 34–42%. Like NOS activity, citrulline synthesis was 23% lower ($P < 0.05$) in placenta of high line gilts compared with low line gilts and did not differ ($P > 0.05$) in endometrium between both lines of gilts. Placental or endometrial citrulline synthesis from arginine did not differ ($P > 0.05$) between d 40 and d 60 of gestation.

Ornithine decarboxylase activity. Maternal dietary protein deficiency decreased ($P < 0.05$) ODC activities in placenta and endometrium by 44–47% (**Table 5**). No difference was detected ($P > 0.05$) for placental or endometrial ODC activity between low line and high line gilts. Placental and endometrial ODC activities decreased ($P < 0.05$) by ~55% at d 60 compared with d 40 of gestation.

Tissue polyamine concentrations. **Table 6** summarizes concentrations of putrescine, spermidine and spermine in placenta and endometrium. The concentrations of their N^1-acetyl derivatives and cadaverine in placenta and endometrium were close to or below the detection limit of our HPLC

TABLE 3

Nitric oxide synthase activities in placenta and endometrium of gilts[1]

Enzyme activity	Dietary protein 0.5% (*n* = 10)	Dietary protein 13% (*n* = 12)	Gilts Low line[2] (*n* = 11)	Gilts High line[3] (*n* = 11)	Gestational age d 40 (*n* = 12)	Gestational age d 60 (*n* = 10)
			nmol/(h · mg protein)			
			Placenta			
Total NOS	1.95 ± 0.15	3.26 ± 0.20*	3.05 ± 0.27	2.27 ± 0.22**	2.50 ± 0.24	2.86 ± 0.31
iNOS	0.83 ± 0.08	1.70 ± 0.13*	1.54 ± 0.18	1.07 ± 0.14**	1.18 ± 0.16	1.45 ± 0.19
cNOS	1.12 ± 0.10	1.61 ± 0.10*	1.60 ± 0.12	1.18 ± 0.09**	1.35 ± 0.12	1.41 ± 0.12
			Endometruim			
Total NOS	1.24 ± 0.12	2.02 ± 0.14*	1.88 ± 0.19	1.46 ± 0.15	1.50 ± 0.16	1.86 ± 0.20
iNOS	0.50 ± 0.04	0.77 ± 0.06*	0.71 ± 0.07	0.58 ± 0.06	0.62 ± 0.07	0.66 ± 0.06
cNOS	0.74 ± 0.08	1.26 ± 0.08*	1.16 ± 0.12	0.89 ± 0.09	0.87 ± 0.10	1.19 ± 0.13

* $P < 0.05$, different from the 0.5% protein group; ** $P < 0.05$, different from the low line gilts.
[1] Data are means ± SEM with the number of gilts in parentheses.
[2] Genetically low plasma concentrations of total cholesterol.
[3] Genetically high plasma concentrations of total cholesterol.

TABLE 4

Synthesis of citrulline from arginine by nitric oxide synthases in incubated placenta and endometrium of gilts[1]

	Dietary protein		Gilts		Gestational age	
Tissue	0.5% (n = 10)	13% (n = 12)	Low line[2] (n = 11)	High line[3] (n = 11)	d 40 (n = 12)	d 60 (n = 10)
			pmol/(h · g wet tissue)			
Placenta	302 ± 21	519 ± 29*	475 ± 38	366 ± 31‡	421 ± 41	420 ± 46
Endometrium	242 ± 13	367 ± 16*	332 ± 27	289 ± 19	296 ± 25	328 ± 24

[1] Data are means ± SEM with the number of gilts in parentheses.
[2] Genetically low plasma concentrations of total cholesterol.
[3] Genetically high plasma concentrations of total cholesterol.
* $P < 0.05$, different from the 0.5% protein group; ** $P < 0.05$, different from the low line gilts.

method and were therefore not determined in this study. Maternal dietary protein deficiency decreased ($P < 0.05$) the concentrations of putrescine, spermidine and spermine in placenta and endometrium by 28–44%. Placental or endometrial polyamine concentrations did not differ ($P > 0.05$) between low line and high line gilts. Placental and endometrial polyamine concentrations decreased ($P < 0.05$) by 28–44% at d 60 compared with d 40 of gestation.

DISCUSSION

The placenta and uterus undergo rapid formation of new blood vessels (angiogenesis) and marked growth during pregnancy (Reynolds et al. 1992). In the pig, which possesses a noninvasive, diffuse type of epithelio-chorial placentation, placental development is maximal by d 60 of gestation (Knight et al. 1977). Angiogenesis during early gestation is necessary to increase uterine and placental-fetal blood flow and therefore the supply of nutrients from maternal to fetal blood (Ford 1995). Thus, placental growth is a critical factor for controlling intrauterine fetal growth (Ott et al. 1997, Reynolds and Redmer 1995). Physiological concentrations of polyamines and NO (products of L-arginine) are key regulators of angiogenesis (Takigawa et al. 1990, Ziche et al. 1994). Placental and uterine growth are also dependent on ODC activity (Williams and McAnulty 1976), and endogenous polyamine synthesis is essential to early mammalian embryogenesis (Fozar et al. 1980). In addition, NO plays an important role in regulating vascular tone of uterine and placental-fetal vessels (Sooranna et al. 1995), and endogenous NO synthesis is necessary for ovulation (Shukovski and Tsafriri 1994), fertilization (Herrero et al. 1996) and successful embryonic attachment and development in the uterus (Gouge et al. 1998, Novaro et al. 1997). Thus, NOS and ODC play critical roles in embryonic, placental and fetal development. This is consistent with the unusual abundance of both arginine and ornithine in porcine allantoic fluid at d 40 and 60 of gestation (Wu et al. 1996a).

Although there have been studies of effects of maternal food restriction on placental ODC activity in rats (Williams and McAnulty 1976), there is little information regarding the effect of maternal dietary protein deficiency on placental or endometrial ODC activity. Also, data are not available regarding effects of maternal protein malnutrition on placental or endometrial concentrations of free amino acids and polyamines. Likewise, little is known about regulation of placental or endometrial NOS activity by maternal nutrition. Three novel findings from this study deserve emphasis. First, arginine concentrations (Table 2) and total NOS activity (Table 3) in placenta and endometrium were lower in protein-deficient gilts compared with gilts fed the 13% protein diet. The decrease in total NOS activities in placenta and endometrium of protein-deficient gilts resulted from the reduction in both cNOS and iNOS activities. Consistent with these results, NO synthesis from arginine (as measured by citrulline generation) was lower in placenta and endometrium of protein-deficient gilts compared with control gilts (Table 4). Second, ornithine concentrations (Table 2) and ODC activity (Table 5) decreased remarkably in placenta and endometrium of protein-

TABLE 5

Ornithine decarboxylase activity in placenta and endometrium of gilts[1]

	Dietary protein		Gilts		Gestational age	
Tissue	0.5% (n = 10)	13% (n = 12)	Low line[2] (n = 11)	High line[3] (n = 11)	d 40 (n = 12)	d 60 (n = 10)
			nmol/(h · mg protein)			
Placenta	0.75 ± 0.10	1.34 ± 0.16*	0.91 ± 0.16	1.22 ± 0.19	1.43 ± 0.17	0.62 ± 0.05§
Endometrium	0.54 ± 0.04	1.01 ± 0.14*	0.80 ± 0.14	0.76 ± 0.11	1.05 ± 0.13	0.46 ± 0.03§

[1] Data are means ± SEM with the number of gilts in parentheses.
[2] Genetically low plasma concentrations of total cholesterol.
[3] Genetically high plasma concentrations of total cholesterol.
* $P < 0.05$, different from the 0.5% protein group; ** $P < 0.05$, different from the d 40 group.

TABLE 6

Concentrations of polyamines in placenta and endometrium of gilts[1]

Polyamine	Dietary protein		Gilts		Gestational age	
	0.5% (n = 10)	13% (n = 12)	Low line[2] (n = 11)	High line[3] (n = 11)	d 40 (n = 12)	d 60 (n = 10)
			nmol/g wet tissue			
			Placenta			
Putrescine	52 ± 4	82 ± 8*	64 ± 7	73 ± 9	79 ± 9	56 ± 3**
Spermidine	118 ± 13	178 ± 21*	131 ± 16	169 ± 24	197 ± 20	94 ± 6**
Spermine	143 ± 11	219 ± 25*	168 ± 19	201 ± 28	227 ± 24	134 ± 8**
			Endometrium			
Putrescine	37 ± 3	66 ± 9*	51 ± 7	55 ± 8	67 ± 9	36 ± 2**
Spermidine	99 ± 8	152 ± 16*	124 ± 18	132 ± 17	165 ± 15	83 ± 5**
Spermine	116 ± 10	162 ± 18*	149 ± 17	133 ± 16	177 ± 13	97 ± 7**

* $P < 0.05$, different from the 0.5% protein group; ** $P < 0.05$, different from the d 40 group.
[1] Data are means ± SEM with the number of gilts in parentheses.
[2] Genetically low plasma concentrations of total cholesterol.
[3] Genetically high plasma concentrations of total cholesterol.

deficient gilts compared with gilts fed the 13% protein diet, resulting in decreased concentrations of putrescine, spermidine and spermine (Table 6). Third, placental and endometrial ODC activities and polyamine concentrations decreased at d 60 compared with d 40 of gestation. This suggests that relatively high rates of polyamine synthesis in placenta and endometrium precede maximal placental development (d 60 of gestation) in the pig (Knight et al. 1977). In the rat, placental ODC activity and polyamine concentrations were greatest at the time of maximal placental development and declined rapidly thereafter (Guha and Janne 1976, Hoshiai et al. 1981). Previous studies established that low line and high line gilts differed in plasma metabolites (e.g., total cholesterol, HDL-cholesterol, triglycerides and alkaline phosphatase) (Pond et al. 1997) and in reproductive performance (Wise et al. 1993). Litter size (number of fully formed pigs born per litter) and ovulation rate (number of corpora lutea at d 60 of pregnancy) decreased in high line gilts compared with low line gilts (Wise et al. 1993). Our recent study suggested that fetal amino acid concentrations differed between low line and high line gilts (Wu et al. 1998). Results of the present study also revealed differences in placental activities of NOS between low line and high line gilts. The reduction in total NOS activities in placenta of high line gilts resulted from decreases in both cNOS and iNOS activities (Table 3). Consistent with the enzyme assay, placental NO generation from L-arginine was lower in high line gilts compared with low line gilts (Table 4). It is noteworthy that hypercholesterolemia is also associated with impaired NO synthesis in rabbits (Cooke et al. 1992) and in humans (Creager et al. 1992) by yet unknown mechanisms. Whether impaired placental NO synthesis during early gestation is responsible for the decreased reproductive performance in high line gilts (Wise et al. 1993) warrants further investigation.

Results of this study may help to understand the mechanism responsible for retarded placental and fetal growth induced by maternal dietary protein deficiency. Because maternal uterine arterial plasma concentrations of basic and neutral amino acids did not differ between control and protein-deficient gilts (Wu et al. 1998), decreased concentrations of these amino acids in placenta and endometrium (Table 5) and in fetal umbilical venous plasma (Wu et al. 1998) suggest that dietary protein deficiency impairs amino acid transport by these two organs, as shown for placenta of protein-deficient pregnant rats (Malandro et al. 1996). A decrease in tissue concentrations of amino acids (particularly essential amino acids) (Appendixes 1 and 2) will lead to decreased protein synthesis and the expression of NOS and ODC in placenta and endometrium. In addition, decreases in both arginine concentrations and NOS activity result in reduced NO production by placenta and endometrium (Table 4). Similarly, decreases in both ornithine concentrations and ODC activity resulted in decreased synthesis and concentrations of polyamines (putrescine, spermidine and spermine) in placenta and endometrium (Table 6). On the basis of current knowledge of the biology of NO and polyamines (Gouge et al. 1998, Novaro et al. 1997, Sooranna et al. 1995, Ziche et al. 1994), decreased synthesis of NO and polyamines may impair placental and endometrial angiogenesis and growth. This will lead to a reduction in placental-fetal blood flow, nutrient supply from maternal to fetal blood, conceptus development and ultimately fetal growth retardation.

In conclusion, maternal dietary protein deficiency decreased *1)* concentrations of free arginine and ornithine, *2)* NOS activity and NO synthesis, and *3)* ODC activity and polyamine concentrations in placenta and endometrium of gilts during early gestation. Decreases in NO and polyamine synthesis may impair placental and endometrial angiogenesis and growth, which leads to reduction in placental-fetal blood flow, nutrient supply from the mother to the fetus and ultimately fetal growth retardation. Likewise, a decrease in placental NO synthesis may contribute to altered conceptus development in high line gilts.

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APPENDIX 1

Concentrations of free amino acids in placenta of gilts[1]

Amino acid	Dietary protein		Gilts		Gestational age	
	0.5% (n = 10)	13% (n = 12)	Low line[2] (n = 11)	High line[3] (n = 11)	d 40 (n = 12)	d 60 (n = 10)
	nmol/g wet tissue					
Ala	665 ± 10	804 ± 14*	747 ± 28	735 ± 22	752 ± 26	730 ± 24
Arg	202 ± 16	258 ± 7*	230 ± 12	236 ± 10	227 ± 11	240 ± 13
Asn	49 ± 2	48 ± 2	50 ± 2	48 ± 2	50 ± 2	49 ± 2
Asp	231 ± 8	240 ± 9	234 ± 7	239 ± 9	236 ± 7	238 ± 9
Cit	22 ± 1	21 ± 1	21 ± 1	22 ± 1	21 ± 1	21 ± 1
Gln	791 ± 13	930 ± 12*	860 ± 22	874 ± 28	879 ± 33	854 ± 21
Glu	1060 ± 38	1071 ± 80	1048 ± 33	1083 ± 75	1094 ± 69	1037 ± 54
Gly	379 ± 10	432 ± 12*	417 ± 15	398 ± 18	416 ± 20	401 ± 17
His	58 ± 3	59 ± 3	57 ± 3	59 ± 3	59 ± 3	58 ± 4
Ile	272 ± 7	319 ± 8*	297 ± 9	310 ± 10	297 ± 8	291 ± 9
Leu	152 ± 6	183 ± 7*	171 ± 7	167 ± 5	168 ± 5	171 ± 6
Lys	409 ± 9	493 ± 10	457 ± 14	453 ± 19	466 ± 16	445 ± 20
Met	58 ± 2	60 ± 3	62 ± 2	56 ± 3	61 ± 2	58 ± 3
Orn	118 ± 6	170 ± 6*	150 ± 10	142 ± 10	145 ± 10	148 ± 11
Phe	48 ± 2	46 ± 2	49 ± 3	46 ± 2	49 ± 2	45 ± 2
Pro	314 ± 8	371 ± 11*	344 ± 10	346 ± 12	351 ± 9	338 ± 12
Ser	372 ± 9	423 ± 7	405 ± 9	396 ± 13	402 ± 8	399 ± 14
Taurine	305 ± 11	372 ± 9*	336 ± 14	347 ± 12	345 ± 15	337 ± 11
Thr	209 ± 5	248 ± 7*	232 ± 8	229 ± 9	236 ± 7	230 ± 9
Trp	96 ± 6	98 ± 7	95 ± 8	98 ± 5	95 ± 6	99 ± 7
Tyr	52 ± 4	55 ± 3	55 ± 4	53 ± 3	55 ± 3	52 ± 3
Val	193 ± 6	231 ± 8*	212 ± 9	215 ± 12	217 ± 13	211 ± 15

* $P < 0.05$, different from the 0.5% protein group.
[1] Data are means ± SEM with the number of gilts in parentheses.
[2] Genetically low plasma concentrations of total cholesterol.
[3] Genetically high plasma concentrations of total cholesterol.

APPENDIX 2

Concentrations of free amino acids in endometrium of gilts[1]

Amino acid	Dietary protein		Gilts		Gestational age	
	0.5% (n = 10)	13% (n = 12)	Low line[2] (n = 11)	High line[3] (n = 11)	d 40 (n = 12)	d 60 (n = 10)
	nmol/g wet tissue					
Ala	780 ± 13	889 ± 17*	851 ± 24	827 ± 26	855 ± 27	820 ± 25
Arg	180 ± 9	237 ± 6*	205 ± 13	208 ± 12	227 ± 11	240 ± 10
Asn	45 ± 2	46 ± 2	47 ± 2	44 ± 2	46 ± 2	44 ± 2
Asp	423 ± 12	433 ± 10	421 ± 10	436 ± 12	418 ± 11	430 ± 14
Cit	21 ± 1	23 ± 1	21 ± 1	22 ± 1	22 ± 1	21 ± 1
Gln	380 ± 10	454 ± 14*	421 ± 14	418 ± 17	425 ± 19	419 ± 18
Glu	1293 ± 74	1275 ± 56	1304 ± 54	1261 ± 69	1246 ± 65	1327 ± 82
Gly	889 ± 25	1018 ± 32*	952 ± 28	967 ± 21	978 ± 25	945 ± 27
His	86 ± 5	94 ± 6	93 ± 5	87 ± 7	95 ± 6	85 ± 5
Ile	305 ± 6	371 ± 7*	338 ± 12	345 ± 13	349 ± 14	332 ± 12
Leu	174 ± 5	208 ± 6*	189 ± 7	195 ± 8	197 ± 8	187 ± 6
Lys	357 ± 10	423 ± 12	392 ± 16	395 ± 15	382 ± 15	397 ± 14
Met	51 ± 2	52 ± 2	53 ± 3	50 ± 2	53 ± 3	49 ± 2
Orn	106 ± 6	142 ± 7*	127 ± 9	124 ± 10	122 ± 8	130 ± 9
Phe	40 ± 2	44 ± 2	41 ± 3	43 ± 2	42 ± 3	42 ± 2
Pro	322 ± 8	387 ± 9*	362 ± 15	350 ± 14	357 ± 16	354 ± 17
Ser	348 ± 8	429 ± 12*	396 ± 17	387 ± 11	391 ± 15	393 ± 19
Taurine	619 ± 18	715 ± 10*	684 ± 16	658 ± 22	663 ± 20	679 ± 18
Thr	223 ± 7	270 ± 8*	254 ± 10	243 ± 8	257 ± 9	240 ± 11
Trp	66 ± 3	68 ± 3	69 ± 3	65 ± 4	70 ± 3	63 ± 3
Tyr	42 ± 2	45 ± 2	44 ± 1	43 ± 2	43 ± 2	44 ± 2
Val	189 ± 5	233 ± 6*	217 ± 10	208 ± 8	215 ± 9	209 ± 11

* $P < 0.05$, different from the 0.5% protein group.
[1] Data are means ± SEM with the number of gilts in parentheses.
[2] Genetically low plasma concentrations of total cholesterol.
[3] Genetically high plasma concentrations of total cholesterol.