AMINO ACIDS, POLYAMINES, AND NITRIC OXIDE SYNTHESIS

IN THE OVINE CONCEPTUS

A Thesis

by

HYUK JUNG KWON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2004

Major Subject: Physiology of Reproduction

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ABSTRACT

Amino Acids, Polyamines, and Nitric Oxide Synthesis
in the Ovine Conceptus. (May 2004)
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The objective of this study was to determine concentrations of amino acids and polyamines as well as nitric oxide (NO) and polyamine synthesis in the ovine conceptus (embryo/fetal and associated placental membrane). Ewes were hysterectomized on Days 30, 40, 60, 80, 100, 120, or 140 of gestation to obtain allantoic and amniotic fluids, intercotyledonary placenta, placentomes and uterine endometrium for the analyses. Alanine, citrulline plus glutamine accounted for about 80% of total α-amino acids in allantoic fluid during early gestation. Serine (16.5 mM) contributed about 60% of total α-amino acids in allantoic fluid on Day 140 of gestation. Maximal ornithine decarboxylase (ODC) and arginase activities and highest rates of polyamine and NO synthesis occured in all tissues on Day 40 of gestation. In ovine allantoic and amniotic fluids, polyamines were most abundant during early (Days 40-60) and late (Days 100-140) gestation, respectively. Activity of guanosine 5'-triphosphate-cyclohydrolase I (GTP-CH), and concentrations of NOS cofactors, tetrahydrobiopterin (BH4) and NADPH (nicotinamide adenine dinucleotide), peaked on Day 40 of gestation in placental and endometrial tissues. In these tissues, NO synthesis was positively correlated with total NOS activity, GTP-CH activity, and concentrations of BH4 and NADPH. The physiological significance of these changes was manifested by undernutrition-induced intrauterine growth retardation (IUGR). Maternal undernutrition (50% of National Research Council nutrient requirements) reduced concentrations of total α-amino acids in fetal

plasma and fluids, and retarded fetal growth at both mid (Day 78) and late (Day 135) gestation. Concentrations of polyamines in fetal fluids were lower in underfed ewes than in control-fed ewes. Realimentation of underfed ewes between Days 78 and 135 of gestation increased concentrations of total α-amino acids and polyamines in fetal plasma and fluids, when compared with non-realimented ewes. Results of these studies demonstrate metabolic coordination among the several integrated pathways to enable high rates of polyamine and NO synthesis in the placenta and endometrium during early pregnancy. Collectively, our findings may have important implications for both IUGR and fetal origins of adult disease.

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CHAPTER I

INTRODUCTION

Amino acids are quantitatively important nutrients for fetal growth and development (Battaglia and Meschia, 1988), because they are building blocks for protein synthesis in the fetus. Amino acids are an important source of energy for the growing fetus (Bell et al., 1989) and are essential to embryonic development and viability (Petters et al., 1990; Lane and Gardner, 1997). They also act as interorgan shuttles for nitrogen or carbon (Christensen, 1990; Battaglia, 1992). Amino acids are also substrates for the synthesis of a wide array of molecules with enormous versatility and importance. For example, nitric oxide (NO) and polyamines (products of arginine catabolism) play a vital role in placental growth and development in rats and both are necessary for placental angiogenesis and development.

The demand for amino acids during fetal life is met primarily by transport systems from maternal to fetal plasma as well as *de novo* synthesis by the placenta and/or the fetus (Cetin et al., 1991). Conceptus (embryo/fetus and associated placental membranes and fluids) development during pregnancy can be affected by nutritional factors. The quantity and quality of amino acids are more important to fetal growth and development (Hoet and Hanson, 1999). In general, the ovine fetus takes up more amino acids than are utilized for protein deposition (Battaglia, 2002). However, little is known about metabolic fate or regulatory roles of amino acids in the ovine fetus and placenta. Likewise, little is known about changes in amino acid concentrations in ovine fetal plasma and placental (amniotic and allantoic) fluids during gestation. In the pig, an unsual abundance of arginine (4-6 mM) was observed in allantoic fluid during early (Day

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This thesis follows the style and format of Molecular Reproduction and Development.

40) gestation, compared with maternal plasma arginine concentrations (0.1-0.15 mM) (Wu et al., 1995a, 1996b). Such information provides an important foundation for studying protein nutrition and metabolism in the fetus. The comparison between fetal and maternal amino acid concentrations provides useful information about dynamic changes in nitrogen exchange between the fetus and dam.

The sheep is a widely used animal model for studying human concpetus development (Lemons et al., 1976; Marconi et al., 1989; Meier et al., 1981; Rice et al., 1987; Moores et al., 1994; Stewart et al., 2000; Battaglia and Regnault, 2001; Osgerby et al., 2002). The sheep has a synepitheliochorial type of placenta in which fetal-placental cotyledons fuse with uterine endometrial caruncles to form placentomes. Ovine placental growth occurs largely in the first half of gestation before the majority of fetal growth that occurs during late gestation (Carter and Myatt, 1995). The placentae of all mammalian species undergo rapid formation of new blood vessels (angiogenesis) and marked growth during gestation (Ford, 1995; Reynolds and Redmer, 2001). Placental growth is the critical factor for controlling the survival, growth, and development of the fetus.

Polyamines (putrescine, spermidine, spermine) are key regulators of angiogenesis, early mammalian embryogenesis in the uterus, regulate the synthesis of DNA and protein, and are essential for cell proliferation and differentiation. NO is a signaling molecule, a major endothelium-derived relaxing factor, a mediator of immune responses, a neurotransmitter, and an angiogenic factor (Reynolds and Redmer, 2001). However, little is known about NO and polyamine synthesis in the placenta of the sheep or any other species.

In the pig, nitric oxide synthase (NOS) activity and NO synthesis during early gestation decreased in response to maternal protein deficiency (Wu et al., 1998b). However, little is known about placental NO and polyamine syntheses or hormonal regulation of these two pathways in sheep or any other species. Usually, amino acids are transported from the mother to the fetus by transport

systems. The dysfunction of amino acid transport systems is associated with preeclampsia in the human (Evans et al., 2003). Reduction in NO production by placenta may contribute to disorders of pregnancy, such as preeclampsia and intrauterine growth retardation (IUGR).

Undernutrition in pregnant women may result from low dietary intake of nutrients owing to either a limited supply of foods or severe nausea and vomiting known as hyperemesis gravidarum (Snell et al., 1998). Epidemiological studies in humans suggest that alterations in fetal nutrition and endocrine status may result in developmental adaptations that permanently change the structure, physiology and metabolism of the offspring, thereby predisposing individuals to cardiovascular, metabolic and endocrine diseases in adult life (Barker and Clark, 1997). Thus, studies of the mechanisms for IUGR brought about by maternal undernutrition have important implications for both human medicine and animal agriculture.

Arginine is an essential precursor for the biosynthesis of a variety of substances with enormous importance. Thus, the present work focuses on two metabolic pathways for arginine catabolism. The first pathway is initiated by arginase, the enzyme which catalyzes the conversion of arginine to ornithine and urea; ornithine is utilized for the synthesis of polyamines by ornithine decarboxylase (ODC). The second pathway is the conversion of arginine to citrulline and NO by NOS. Importantly, arginase and NOS enzyme use arginine as a common substrate (Fig 1.1). Thus, arginase may play an important role in regulating NO and polyamine synthesis in placenta.

The objectives of this research are to determine changes in concentrations of amino acid and polyamines in ovine fetal fluids as well as placental and uterine tissues during normal and maternal undernutritional pregnancy. The findings will provide an important foundation for future studies of the regulation of fetal and placental growth by amino acids under altered nutritional and physiological conditions. New knowledge generated from this research will be beneficial for improving the efficiency of ovine reproduction in

ruminants. This will estabilish the role of arginine as a major precursor for placental polyamine synthesis during gestation, and quantify placental NO synthesis.

Arginine

NO ⟶ Embryogenesis ⟵ Polyamines

Placental angiogenesis and growth

Placental-fetal blood flow

Nutrient and O_2 supplies from the mother to the fetus

Fetal growth and development
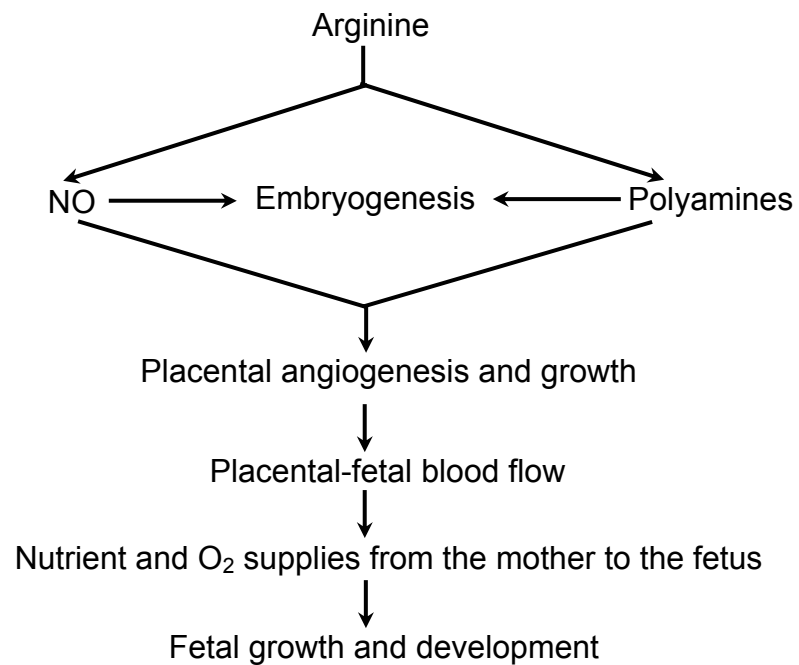
Fig 1.1 Important roles for arginine in embryogenesis as well as placental and fetal growth. Through increases in NO and polyamine synthesis, arginine stimulates angiogenesis and placental blood flow. This auguments the transfer of nutrients and oxygen from the mother to the fetus, thereby promoting fetal growth and development. NO, nitric oxide; O_2, oxygen.

CHAPTER II
LITERATURE REVIEW

Implantation and placentation in sheep

Implantation and placentation are required for fetal growth and development. The first event is that the fertilized ovum reaches the uterus as a morula within 3-4 Days, and then the blastocyst develops and enlarges rapidly. After the initial expantion of the blastocyst at about Day 10, the inner layer forms the yolk sac wall with endoderm, and the elongated chorionic sac contains allantoic cavity that apposes the chorion to the end of the outer chorionic sac and apposes the amnion directly over its central portion (Fig 2.1). Initiation and development of blastocyst cell motility is regulated by amino acid availability, especially leucine and arginine in mouse (Martin et al., 2003). The blastocyst attaches with cytoplasmic protrusions to the endometrial surface, particularly caruncles of endometrium. Caruncles are areas of non-glandular but well vascularized endometrium, and present within the uterus of ruminants.   The allantois originates from the posterior region of the primitive gut, and makes contact with chorion, thus refered to as the chorionallantoic membrane. Patches of chorioallantoic membrane become cotyledons by developing villi, and cotyledons caruncular fuse with uterine epithelium, forming binucleate trophoblasts (Wooding, 1982; Cross et al., 1994). In between the cotyledons is the intercotyledonary chorion, which is covered by simple trophoblast and modified only over the mouths of endometrial glands. Multiple cotyledons are scattered across the placental surface.   Thus, placentation begins with implacntation of the blastocyst beneath the uterine epithelium and differentiation into embryonic and extraembryonic tissues (Cross, 1998)

Placental growth and fetal development in sheep

The placenta is a vital membrane, without which the fetus cannot develop during pregnancy. The placenta is: (1) the interface for the exchange of nutrient
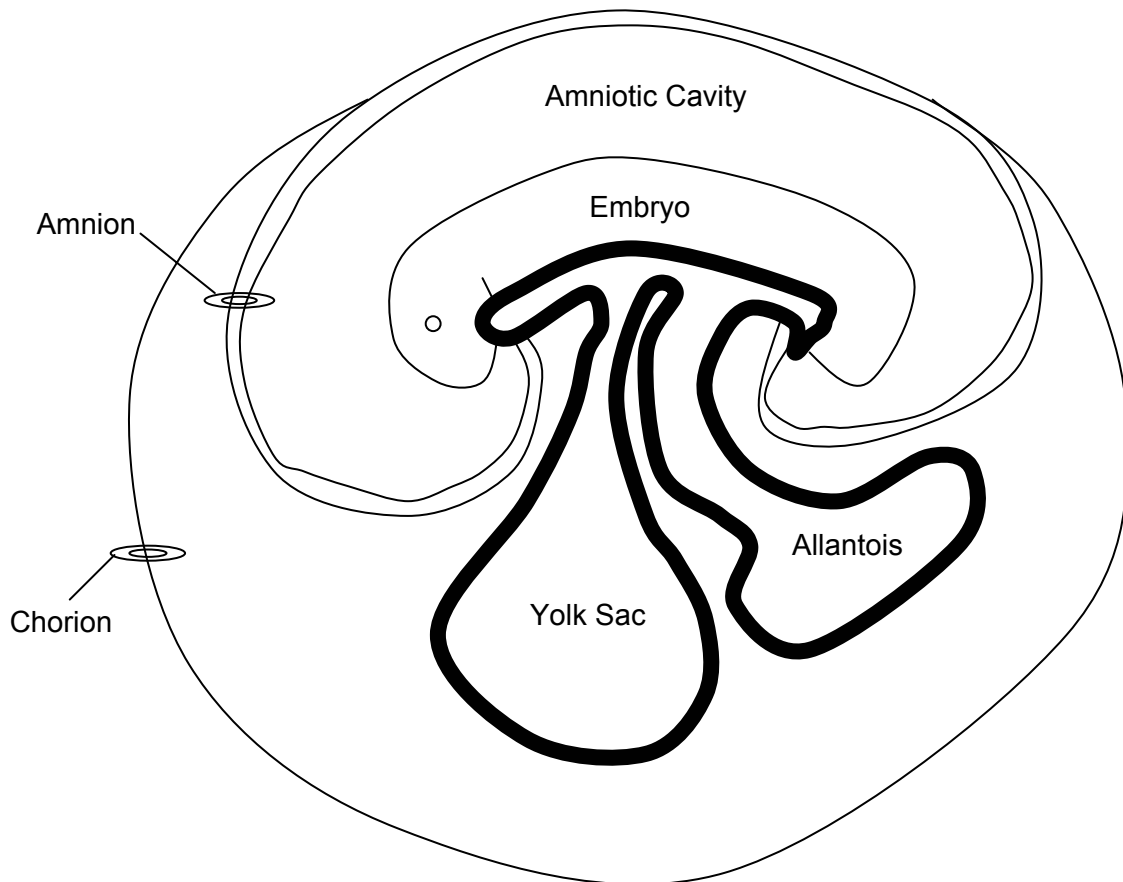
Fig 2.1 Schematic of the ovine conceptus between about Days 20 and 40 of gestation.

and waste products between the mother and the fetus (Rossant and Cross, 2001); (2) the fetal respiratory organ for the exchange of gases of the fetus (Wimsatt,1950; Wooding, 1998; Mossman, 1987); (3) an active endocrine organ that synthesizes and secretes hormones, including progesterone, placental lactogen and growth hormone; and (4) the barrier for the fetus against pathogens and the maternal immune system (Regnault et al., 2002).

The sheep has a synepitheliochorial type of placenta in which fetal-placental cotyledons fuse with uterine endometrial caruncles to form placentomes. Placental angiogenesis during early gestation is necessary to increase placental-fetal blood flow and, therefore, the supply of nutrients from

maternal to fetal blood (Ford, 1995). Placental growth is also a critical factor for controlling the survival, growth and development of the fetus. In sheep, the period of maximal placental weight is attained between Day 30 and 80 of gestation (Schneider, 1996). A positive correlation exists between fetal birth size and placental weight (Heasman et al., 1999). Three critical time points during ovine gestation include: (1) Day 58 (when placentomes are established); (2) placental growth is maximal prior to Days 60 to 70 of gestation; and (3) the maximal fetal growth occurs between Days 70 to 135 (a period of rapid fetal growth) (Osgerby et al., 2002). The endometrium (a mucosal layer underlying the placenta) is also important for early embryonic development, implantation, placentation, as well as successful placenta and fetal development (Bazer, 1992). The developing fetus (Fig 2.1), surrounded by the amniotic fluid compartment and connected with the allantoic sac via the urachus and placental vasculature, receives nutrients mainly via the umbilical vein (Battaglia and Meschia, 1988). The umbilical vein carries oxygen and nutrients obtained from the placenta to the fetus. The amniotic fluid of the amnion provides a unique aqueous environment in which the fetus develops symmetrically (Fig 2.1). Amniotic fluid is composed of water and electrolytes from both the fetus (kidneys, lungs, epidermis, and fetal blood vessels in the placenta and umbilical cord) and the mother (decidual blood vessels via amniotic membranes) (Schmidt, 1992). When it is swallowed, amniotic fluid is a significant source of nutrients for the fetus (Schmidt, 1992). The allantoic sac was traditionally considered to be a reservoir for fetal wastes (Alexander and Williams, 1968). This fluid is derived from fetal and maternal secretions, but primarily from placental transport mechanisms (Bazer, 1989). However, recent studies with pigs have shown that the allantoic sac plays an important role in the accumulation of nutrients and metabolism of both uteroferrin (a progesterone-induced iron-binding protein) and iron (Bazer, 1989), suggesting a hitherto unrecognized function of the allantoic sac in fetal nutrition.

Thus, the placenta and fetus interact in a variety of ways to ensure adequate nutrient supplies to the fetus to support developmental, metabolic, and signaling processes that are unique to fetal growth and development (Hay, 1991). The fetal demand for some factors, e.g. amino acids and glucose, is met in several ways, such as synthesis by the placenta and/or by the fetus and transport systems from maternal to fetal blood. Amino acids, macronutrients, undoubtedly play a crucial role in supporting both placental and fetal growth.

Roles of amino acids in conceptus development

Amino acids are quantitatively important nutrients for many processes involved in fetal growth and development (Battaglia and Meschia, 1988). Amino acids represent the main source of nitrogen for the growing fetus. They are precursors for the synthesis of proteins and other biologically important substances, such as peptides, creatine, carnitine, porphyrins, polyamines, and nitric oxide (Reeds and Hutchens, 1994; Wu and Morris, 1998). Amino acids also function as antioxidants (Fang et al., 2002), regulators of hormone secretion (Kuhara et al., 1991; Flynn et al., 2002), major fuels for fetal growth (Bell et al., 1989), and signaling molecules (Wu and Morris, 1998; Flynn et al., 2002). Some amino acids (e.g. arginine and ornithine) regulate the secretion of insulin, glucagon, growth hormone, prolactin, and placental lactogen. Other amino acids (e.g. leucine and arginine) regulate gene expression and signal transduction. In addition, amino acids are an important source of energy for the growing fetus (Bell et al., 1989).

Glutamine

Glutamine is the physiological precursor of both ornithine and arginine in mammals, including pigs (Wu et al., 1995b) (Fig 2.2). In particular, glutamine serves as an essential precursor for the synthesis of purine and pyrimidine nucleotides for cell division, amino sugars and NAD+ (nicotinamide adenine dinucleotide) (Krebs, 1980) and serves as an important fuel for rapidly dividing

Glutamine

Glutaminase $\rightarrow$ NH$_3$

Glutamate

P5C synthase

Pyrroline-5-carboxylate

Glu

α-KG

OAT

Ornithine

CP

OCT

Citrulline

ASP

ASS

Argininosuccinate

Fumarate

ASL
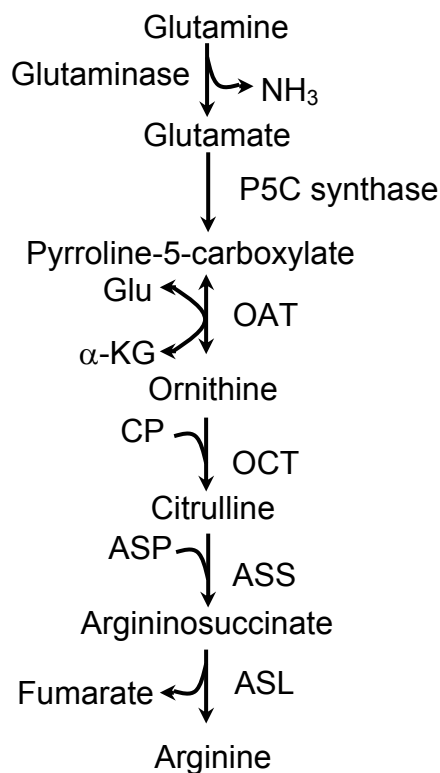
Arginine

Fig 2.2. *De novo* synthesis of tetrahydrobiopterin in cells. ASS, argininosuccinate synthase; ASL, argininosuccinate lyase; ASP, aspartic acid CP, carbamoyl phosphate; Glu, glutamic acid; α-KG, α-ketoglutarate; OAT, ornithine aminotransferase; OCT, ornithine carbamoyltransferase; P5C, pyrroline-5-carboxylate; NH$_3$, ammonia.

cells. In sheep, the maternal to fetal flux of glutamine was the greatest among all amino acids (Bell et al., 1989). Glutamine is essential to embryonic development (Petters et al., 1990) and viability (Lane and Gardner, 1997). The abundance of glutamine in fetal fluids is consistent with its important role in fetal nitrogen and carbon metabolism (Vaughn et al., 1995), as well as in early mammalian embryogenesis (Petters et al., 1990). Glutamate, a product of glutamine hydrolysis by glutaminase, binds with the N-methyl-D-aspartic acid receptor, resulting in an increase in the influx of calcium and its intracellular

concentration and consequently activation of neuroral NOS (nNOS) for NO synthesis (Atlante et al., 2001).

Arginine

Arginine is the precursor of ornithine and NO (Fig 2.3), a free radical with enormous physiological importance, including its possible role in regulating uterine and placental-fetal blood flow development. Although arginine is found in liver via in the urea cycle, there is no net synthesis of arginine by this organ because an exceedingly high activity of arginase results in rapid hydrolysis of arginine (Flynn et al., 2002). Arginine is one of the most abundant amino acids deposited in fetal tissue proteins (Meier et al., 1981; Sparks et al., 1985; Wu et al., 1999a), indicating the quantitative importance of arginine in fetal growth.

In addition, arginine is the immediate physiological precursor of ornithine and NO. NO has been recognized as an endothelium-derived relaxing factor, a neurotransmitter and a modulator of the immune response (Moncada and Higgs,1993). Recent studies also implicate NO as a critical factor in increasing
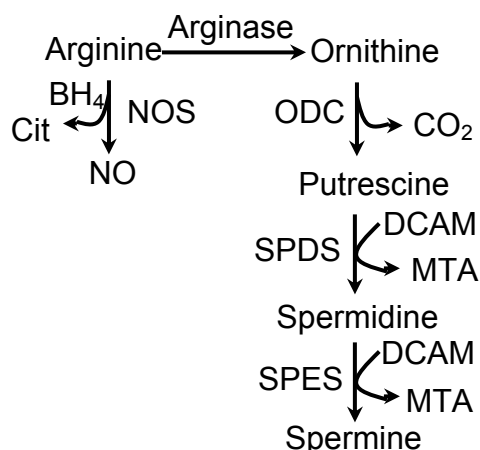
Fig 2.3. Pathways for the production of NO and polyamines from arginine. BH_4, tetrahydrobiopterin; Cit, citrulline; DCAM, decarboxylated S-adenosylmethionine; MTA, methylthioladenosine; NOS, nitric oxide synthase; ODC, ornithine decarboxylase; SPDS, spermidine synthase; SPES, spermine synthase.

Uterine blood flow, and probably placental-fetal blood flow during gestation (Weiner et al., 1994; Suburo et al., 1995). Therefore, the arginine-dependent NO synthesis plays an important role in regulating nutrient supply to the fetus. Moreover, NO has been shown to play a crucial role in maintaining uterine quiescence during pregnancy (Buhimschi et al., 1995).

Ornithine

Ornithine serves as the precursor for biosynthesis of polyamines (Fig 2.3). Ornithine also plays an important role in ammonia detoxification via the hepatic urea cycle (Smith, 1981; Meijer et al., 1990). Half of the nitrogen must be supplied in the form of aspartate and another half in the form of carbamoyl phosphate in the synthesis of urea through the ornithine cycle (Fig 2.3). As amino acid oxidation is a major source of energy for the fetus (Lemons et al., 1976; Bell et al., 1989), the ammonia generated by this process must be efficiently removed as water-soluble urea, because of ammonia toxicity to animal cells (Meijer et al., 1990) and, particularly, the brain (Visek, 1968). There is no known pathway for direct conversion of citrulline into ornithine in animals (Wu and Morris, 1998). Thus, via the formation of arginine from citrulline in cells and tissues, citrulline can be a major source of ornithine, which has versatile roles in cellular nutrition and metabolism. For example, ornithine is the precursor for the synthesis of polyamines, which are essential for placental and fetal development (Fozard et al., 1980).

Citrulline

Citrulline is an effective precursor for the synthesis of arginine. NO is produced from L-arginine by NOS enzymes, forming the free radical NO and citrulline as a co-product. The conversion of citrulline into arginine consumes ammonia in the form of aspartate (Wu and Morris, 1998). All three NOS isoforms use NADPH as an electron donor and employ five enzyme cofactors to

catalyze a five-electron oxidation of arginine to NO with stoichiometric formation of citrulline (Bredt, 1999). L-citrulline is utilized for NO production in virtually all cell types (Su and Austic, 1999). As a neutral amino acid, citrulline does not compete with basic amino acids for transport by cells, because citrulline transporter is not energy-dependent and thus does not use the mitochondrial proton potential gradient (Indiveri et al., 1997). Neutral amino acids share transport systems which are different form the ones responsible for cellular uptake of charged amino acids (Christensen, 1990). Citrulline has recently been recognized as an efficient antioxidant protecting DNA, lipids and proteins from hydroxyl radical-induced oxidative damage (Akaschi et al., 2001). This action of citrulline may contribute to a protective environment for fetal development. In addition, citrulline is an effective precursor for arginine synthesis in virtually all animals because of the widespread presence of argininosuccinate synthase and argininosuccinate lyase in tissues, including placenta and kidneys (Wu and Morris, 1998) (Fig 2.2).

Other amino acids

Important nitrogenous products of amino acid metabolism in animals are summarized in Table 2.1. The branched amino acids (leucine, isoleucine and valine) are used within the fetus and placenta for energy production and protein synthesis. Proline plays an important role in making collagen in vertebrates, and generating of extracellular matrix. Lysine shares the same transport system (y^+) with arginine for entry into cells, and thus an increase in extracellular lysine concentration would be expected to decrease the intracellular availability of arginine for constitutive NO synthesis. Taurine is a β-amino acid critical for neurological and cardiovascular development (Sturman, 1993).

As previously indicated, amino acids play a vital role in development of the conceptus. In general, the ovine fetus takes up more amino acids than utilized for protein deposition. However, little is known about the metabolic fate or regulatory roles of amino acids in the ovine fetus. Likewise, little is known ab-

TABLE 2.1 Important nitrogenous products of amino acid metabolism in animals

Precursors	Products	Functions
Arg	NO	Vasodilator; signaling molecule; angiogenesis; cell metabolism; apoptosis
	Agmatine	Signaling molecule; inhibitor of NOS and ODC; brain & renal function
Cys	Taurine	Antioxidant; muscle contraction; bile acid conjugates; retinal function
Glu	GABA	Neurotransmitter; inhibitor of glutamatergic, serotonin & NEPN activities
Gln	Glu & Asp	Neurotransmitters; fuels for enterocytes; components of the malate shuttle
	Citrulline	Free radical scavenger; arginine synthesis
	Glucosamine	Glycoprotein & ganglioside formation; inhibitor of NO synthesis
	Ammonia	Renal regulation of acid-base balance; CP formation; cell metabolism
Gly	Serine	One-carbon unit metabolism; ceramide & phosphatidylserine formation
	Heme	Hemoproteins (e.g., hemoglobin, myoglobin, Cyt C, Cyt P450 & catalase)
His	Histamine	Allergic reaction; vasodilator; control of gastric acid & central Ach secretion
Met	Homocysteine	Oxidant; inhibitor of NO synthesis; risk factor for cardiovascular disease
	Betaine	Methylation of homocysteine to methionine; one-carbon unit metabolism
	Choline	Synthesis of Ach (neurotransmitter & vasodilator), PTC & betaine
Ser	Glycine	Antioxidant; bile acid conjugates; neurotransmitter; immunomodulatory
Tryptophan	Serotonin	Neurotransmitter; smooth muscle contraction; hemostasis
	NAS	Inhibition of sepiapterin reductase (an enzyme for BH4 synthesis)
	Melatonin	Circadian & circannual rhythms; free radical scavenger; antioxidant
Arg & Met	Polyamines	Gene expression; DNA & protein synthesis; ion channel function; apoptosis; signal transduction; antioxidants; cell function, proliferation & differentiation
Gln & Asp	Nucleic acids	Gene expression; cell cycle and function; protein and uric acid synthesis
Trp & Gln	NAD(P)	Coenzymes for oxidoreductases; substrate of poly (ADP-ribose) polymerase
Arg, Pro or Gln	Ornithine	Glutamate, glutamine & polyamine synthesis; mitochonfrial integrity

Ach, acetylcholine; BH4, tetrahydrobiopterin; CP, carbamoylphosphate; Cyt, cytochrome; EPN, epinephrine; GABA, γ-aminobutyrate; NAS, N-acetylserotonin; NEPN, norepinephrine; NOS, nitric oxide synthase; ODC, ornithine decarboxylase; PTC, phosphatidylcholine; T3, triiodothyronine; T4, thyroxine.

out changes in amino acid concentrations in ovine fetal plasma and placental (amniotic and allantoic) fluids during gestation. Comparisons between fetal and maternal amino acid concentrations provide useful information about dynamic changes in nitrogen exchange between the fetus and dam.

Polyamine synthesis

Arginine from the diet or from protein breakdown is hydrolyzed by arginase to form urea and ornithine. Arginase is a key enzyme of the urea cycle that removes high toxic ammonium ions resulting from the protein degradation (Jenkinson et al., 1997; Wu and Morris, 1998). Type I arginase is a cytosolic enzyme that is most abundant and highly expressed in the liver. On the other hand, Type II arginase is a mitochondrial enzyme that is located primarily in the mitochondrial matrix and is widespread. Type II arginase may be involved primarily in the production of ornithine as a precursor for the synthesis of proline, glutamate, or polyamines. Arginase activity is inhibited by ornithine, lysine, and the branched amino acids (leucine, isoleucine, and valine) (Levillain et al., 1994). By modulating arginine availability, arginase can regulate NO synthesis (Li et al., 2001).

Ornithine serves as the precursor for biosynthesis of polyamines (Fig 2.3), with the first and rate-limiting step being catalyzed by ornithine decarboxylase (ODC) (Johnson, 1988). Ornithine, as mentioned above, plays an important role in ammonia detoxification via the hepatic urea cycle (Smith, 1981). The importance of ornithine and ODC in fetal development is suggested by the previous finding that polyamines are essential to early mammalian embryogenesis (Forzard et al., 1980). Rapid cell proliferation is associated with enhanced ODC activity (Johnson, 1988), which necessitates an increased availability of ornithine. The activity of ODC is dramatically increased in rapid tissue growth (Janne et al., 1978) and embryogenesis in rats (Russel and McVicker, 1972; Guha and Janne, 1976). In rat placenta, the ODC activity

increased 40-fold from Day 10 to 17 of gestation and at the same time paralleled the period of rapid growth of the placenta (Hoshiai et al., 1981).

The polyamines (putrescine, spermidine and spermine) are cationic molecules present in all living organisms. Many natural products contain polyamine residues. Polyamines are synthesized from ornithine (a product of arginine catabolism) and are essential to cell growth and diffentiation. For example, uterine and placental growth is dependent on ODC activity (Willams and McAnulty, 1976). Thus, ODC is widely expressed in all cell types, and expression of ODC is regulated by a variety of hormones and cytokines (Wu and Morris, 1998). On the basis of recent findings of intestinal polyamine synthesis (Wu et al., 2000a, 2000b), ornithine produced in placental mitochondria can be available for polyamine synthesis by cytosolic ODC, spermidine synthase, and spermine synthase (Fig 2.3).

Polyamines (putrescine, spermidine, and spermine) are key regulators of angiogenesis, early mammalian embryogenesis, placental trophoblast growth, and embryonic development in the uterus (Forzard et al., 1980; Henningsson et al., 1982). Through binding intracellular RNA, DNA, nucleotide triphosphates, proteins, and other negatively charged molecules, polyamines (polycationic molecules) regulate gene expression, signal transduction, ion channel function, as well as DNA and protein synthesis (Igarashi and Kashiwagi, 2000). Polyamines, particularly spermine, are also endogenous scavengers of reactive oxygen species and hydroxyl radical (Ha et al., 1998), thereby protecting DNA (Khan et al., 1992; Basu and Marton, 1995), proteins, and lipids from oxidative damage (Chattopadhray et al., 2003), and potential transformation (Ha et al., 1998). Particularly, spermidine has an effect on the elongation of nascent RNA chains in cell nucleus (Hoshiai et al., 1981). Thus, polyamines are essential for cell proliferation, differentiation, and function. Consequently, the deficiency of ODC results in the depletion of polyamines and leads to massive apoptosis in the inner cellular membrane (Pendeville et al., 2001).

NO or not, does it matter during pregnancy?

NO is derived from the amino acid L-arginine in an oxidative reaction that consumes molecular oxygen and NADPH (nicotinamide adenine dinucleotide phosphate hydrogen). NOS is a monooxygenase that converts L-arginine and molecular oxygen to NO and L-citrulline. NOS requires FAD (flavin adenine dinucleotide), heme, calcium, FMN (flavin mononucleotide), calmodulin and BH_4 (tetrahydrobiopterin) as cofactors, and the products of this reaction are NO, $NADP^+$ and citrulline. The regulated synthesis of NO in biological systems is essential, because NO cannot be stored and it is released as needed. NO can diffuse and permeate across membranes, without the need for specific receptors. Interaction of NO with oxygen results in the formation of the relatively inactive end products nitrate (NO_3^-) and nitrite (NO_2^-). There are 3 isoforms of NOS identified: neuronal NOS (nNOS or type I); inducible NOS (iNOS or type II); and endothelial NOS (eNOS or type III). Those three NOS isoforms can be divided into two functional classes based on the dependency of the enzymes on calcium for their activity. Inducible forms, Type II NOS or iNOS, contain irreversibly bound calmodulin, and are hence largely independent of Ca^+, although the constitutive forms, Type I NOS and III NOS (collectively referred to as cNOS), require Ca^+ for their activity.

Constitutive NOS is constitutively expressed in various cell types. In contrast, inducible NOS is induced by inflammatory cytokines and certain hormones (Knowles and Moncada, 1994). The NOS isoforms are present in the rat uterus during gestation (Buhimschi et al., 1996) and also in uterine tissues in sheep (Sladek et al., 1997). NOS expression or activity exists in myometrium and endometrium of pregnant and non-pregnant sheep (Figueroa and Massmann, 1995; Massmann et al., 1999). Uterus can have the capacity to produce NO, and NOS activity is high during pregnancy and decreases at the onset of parturition (Natuzzi et al., 1993). Also uterine NO derives from neuronal NOS and endothelial NOS during the last third of gestation (Massmann et al.,

1999). On the other hand, the most abundant NOS mRNA in the rat uterus is the inducible NOS isoform (Ali et al., 1997).

Nitric oxide production in different species

Rates of NO production vary among tissues, depending on NOS activity and the availability of its substrates and cofactors. In humans, NOS activity in placenta is greater than that in other tissues (Di Iulio et al., 1996; Ramsay et al., 1996; Thomson et al., 1997). In rats, inducible NOS and endothelial NOS were present in uterus during gestation (Buhimschi et al., 1996) and inducible NOS expression was not detected on Day 22 of pregnancy (Farina et al., 2001). Constitutive NOS activity was higher than inducible NOS activity in rat placenta during gestation, and thus NO production in placenta was higher during early gestation compared to mid- and late gestation (Thanda et al., 1996). This result is consistent with the report that the activity of Ca^{2+}-dependent NOS increased during early gestational age (Weiner and Thompson, 1997).

Both constitutive NOS and inducible NOS are expressed in porcine placenta on Days 40 to 60 of gestation (Wu and Morris, 1998). Placental NOS activity and NO synthesis were decreased during early gestation in response to maternal protein deficiency in pig (Wu et al. 1998b). In sheep, there is very little inducible NOS activity in nonpregnant uterus of sheep (Figueroa and Massmann, 1995; Massmann et al., 1999). Inducible NOS activity may account for approximately 10% of total NOS activity in the endometrium (Massmann et al., 1999). However, constitutive NOS activity significantly increased in ovine myometrium, but not endometrium, in response to estrogen administration (Figueroa and Massmann, 1995). In sheep, NO production by cotyledonary and intercotyledonary placenta tissues increased between Day 110 and 130 (Zheng et al., 2000). However, little is known about placental and endometrium NO synthesis during early gestation.

As previously indicated, NO, synthesized from L-arginine, has enormous metabolic versatility and physiological importance, including its potential roles in

regulating placental angiogenesis (Reynolds and Redmer, 2001) and uterine blood flow during gestation (Sladek et al., 1997). NO may play a critical role in regulating uterine blood flow and thus nutrient supply to the fetus during gestation (Sladek et al., 1997). However, as the endothelial vasodilator (Magness et al., 1996) and nitrovasodilator (Zhang et al., 2001), NO plays an important role in conceptus development. Other physiological roles of NO during gestation are to regulate placental blood flow and limit platelet aggregation at the interface between the uterine and fetoplacental circulation (Norman and Cameron, 1996). During pregnancy, arginase (Lowe, 2000), cyclooxygenase (Faletti et al., 1999), or sex hormones (Weiner et al., 1994) regulate placental NO synthesis.

At present, little is known about NO synthesis or its regulation in the ovine placenta. Available evidence supports the ideas that NO produced in placenta during pregnancy can: (1) regulate maintenance of uterine quiescence (Natuzzi et al., 1993; Massmann et al., 1999); (2) maintain or regulate vessel relaxation; (3) control vascular tone (King et al., 1995); (4) limit platelet aggregation in both maternal and fetal circulations (Radomski et al., 1990); and (5) suppress contractions of the underlying myometrium. The reduction of NO production by placenta may contribute to disorders of pregnancy such as pre-eclampsia and intrauterine growth restriction (Sladek et al., 1997).

Studies in intrauterine growth retarded (IUGR) pregnancies

In normal pregnancy, concentrations of amino acids are significantly higher in the fetus than in the mother (McIntosh et al., 1984, Wu et al., 1998a). However, concentrations of amino acids were reduced in fetal rat plasma (Malandro et al., 1996) as well as plasma and fluids of fetal pigs (Wu et al., 1998a) in response to maternal protein deficiency. Under intrauterine growth retardation (IUGR), concentrations of amino acids, such as the branched amino acids (leucine, isoleucine, and valine) (Cetin et al., 1988, 1990) were significantly reduced in ovine fetus compared to normal pregnancy during

second and third trimesters (Cetin et al., 1992a). Cetin and his coworkers suggested impairment in placental amino acid transport in women with IUGR, on the basis the finding that concentrations of the three branched amino acids, which share a common transport system, designated system "L", in the human placenta, were lower in fetal plasma than in maternal plasma (Cetin et al., 1988, 1990, 1992a). Also concentrations of other amino acids, such as arginine, glutamine, and threonine, were consistently reduced in IUGR fetus, whereas, concentrations of some amino acids, particular glycine and proline were increased in amniotic fluid (Cetin et al., 1988; Economides et al., 1989).

Each amino acid has its own unique metabolic pathway in a cell- and tissue-specific manner (Stipanuk and Watford, 2000), but the metabolic fate of amino acids may be different in IUGR. For instance, glycine is usually rapidly taken up by the placenta from the maternal circulation via the "L" system, whose characteristics are Na+ independent. The "L" system is responsible for the transport of several neutral amino acids (Jasson, 2001). Interestingly, glycine is produced in the ovine placenta from serine (Cetin et al., 1991, 1992b; Moores et al., 1994), but metabolism of glycine and serine may be different in IUGR (Cetin et al., 1991, 1992b).

Available evidence indicates that the birth weight and placental weight in sheep were reduced in IUGR (De Barro et al., 1992; Mellor and Murrary, 1981). Fetal growth depends on the supply of nutrients and oxygen from maternal to fetal blood via the placenta. The limitation in the supply of these substances from maternal to fetal plasma may be related to placental dysfunction. Therefore, a decrease in the availability of amino acids in the fetus may contribute to IUGR. Because IUGR is a major health problem in the U.S. and worldwide, elucidating the mechanisms responsible for IUGR is essential to improve pregnancy outcomes, and reduce the high costs of health care associated with small-for-gestation infants.

In summary, available evidence indicates that both NO and polyamines (products of arginine catabolism) are key regulators of angiogenesis,

mammalian embryogenesis, as well as placental and fetal growth. NO is an endothelium-derived relaxing factor, and plays an important role in regulating placental-fetal blood flow, and thus the transfer of nutrients and O_2 from mother to fetus. Recent studies have shown that NO is a key mediator of various female reproductive processes, including implantation, pregnancy maintenance, and delivery. Likewise, polyamines regulate DNA & protein synthesis and, therefore, cell proliferation and differentiation, as well as placental and fetal growth. Thus, amino acids play a crucial role in fetal growth and development. Maternal undernutrition results in IUGR in humans and domestic animals. However, the underlying mechanism(s) remain elusive. The prevalence of maternal undernutrition in the United States and worldwide and the resulting poor pregnancy outcomes have provided an impetus for us to identify means to reverse the fetal growth retardation caused by this condition. New knowledge about the mechanisms controlling fetal growth and development will be beneficial for designing new preventative and therapeutic interventions to optimize intrauterine growth. Promoting an optimal intrauterine environment will not only ensure optimal fetal development but will also reduce the risk of chronic diseases in adult life. This, in turn, will help ensure optimal pregnancy outcomes while saving billions of healthcare dollars annually both in the U.S. and around the world.

CHAPTER III
DEVELOPMENTAL CHANGES OF AMINO ACIDS
IN OVINE FETAL FLUIDS

Introduction

Amino acids serve as essential precursors for the synthesis of proteins, peptides, neurotransmitters, aminosugars, purine and pyrimidine nucleotides, creatine, carnitine, porphyrins, melatonin, melanin, sphingolipids, polyamines, and nitric oxide (Wu and Morris, 1998; Stipanuk and Watford, 2000). Amino acids also function as antioxidants (Fang et al., 2002), regulators of hormone secretion (Kuhara et al., 1991; Flynn et al., 2002), major fuels for fetal growth (Bell et al., 1989), and signaling molecules (Wu and Morris, 1998; Flynn et al., 2002). In particular, glutamine plays an important role in fetal nitrogen and carbon metabolism (Vaughn et al., 1995). Polyamines, synthesized from ornithine (ultimately arginine) by ornithine decarboxylase, are essential to placental development and mammalian embryogenesis (Fozard et al., 1980). Importantly, nitric oxide, synthesized from L-arginine, has enormous metabolic versatility and physiological importance, including potential roles in regulating placental angiogenesis (Reynolds and Redmer, 2001) and uterine blood flow during gestation (Sladek et al., 1997). Furthermore, serine and glycine are a major source of one-carbon units for cellular metabolism, including DNA synthesis and methylation (Snell and Fell, 1990). Thus, amino acids play a vital role in development of the conceptus (embryo/fetus and associated placental membranes). The developing fetus, surrounded by the amniotic fluid compartment and connected with the allantoic sac via the urachus and placental

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vasculature, receives nutrients mainly via the umbilical vein (Battaglia and Meschia, 1988). The amniotic fluid provides a unique aqueous environment in which the fetus develops symmetrically. When it is swallowed, amniotic fluid is a significant source of nutrients for the fetus (Schmidt, 1992). In contrast, the allantoic sac was traditionally considered to be a reservoir for fetal wastes (Alexander and Williams, 1968). However, recent studies with pigs have shown that the allantoic sac plays an important role in the accumulation of nutrients and metabolism of both uteroferrin (a progesterone-induced, iron-binding protein) and iron (Bazer, 1989), suggesting a hitherto unrecognized function of the allantoic sac in fetal nutrition.

We recently reported an unusual abundance of arginine (4-6 mM) in porcine allantoic fluid during early (Day 40) gestation, compared with maternal plasma arginine concentrations (0.1-0.15 mM) (Wu et al., 1995a, 1996a, 1998a). However, it is not known whether such high concentrations of arginine are unique for porcine allantoic fluid or whether they represent an important physiological phenomenon for mammals. Although there have been studies of amino acid uptake into umbilical vessels (Lemons et al., 1976), placental arginine transfer (Thureen et al., 2002), as well as placental and hepatic metabolism of glycine, serine, glutamate and glutamine (Geddie et al., 1996; Chung et al., 1998) in fetal sheep during late gestation (Days 118–146), little is known regarding changes in concentrations of amino acids in ovine fetal fluids associated with conceptus development. Such information will provide a critical database for future studies to quantify amino acid metabolism in the ovine fetus, to define fetal amino acid requirements, and to elucidate mechanisms responsible for intrauterine growth retardation and fetal origin of adult diseases.

We hypothesized that arginine was the most abundant amino acid in ovine allantoic fluid during early gestation. This hypothesis was tested by quantifying concentrations of 24 amino acids in ovine amniotic and allantoic fluids, as well as fetal and maternal plasma, between Days 30 and 140 of

gestation. The measurement of amino acids other than arginine is necessary to determine its relative abundance in ovine fetal fluids.

Material and Methods

Chemicals

High-performance liquid chromatography (HPLC)-grade water and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals used for amino acid analysis were purchased from Sigma Chemicals (St. Louis, MO).

Ewes

Columbia cross-bred ewes were mated to Suffolk rams when detected as being in estrus (Day 0) and at 12 and 24 h later. Ewes were then assigned randomly to be hysterectomized (n = 4 per day) on either Day 30, 40, 60, 80, 100, 120, or 140 of gestation to allow collection of fetal fluids as well as maternal and fetal blood. These gestational ages were selected to include early, mid- and late gestation in sheep (term, 147 days). In preliminary studies, we determined that concentrations of amino acids in fetal plasma, allantoic fluid, or amniotic fluid were similar between twin fetal lambs from the same ewes. Thus, samples were obtained from only one fetus per ewe for amino acid analysis when twin fetal lambs were present. None of the ewes in the study had more than twin fetuses. Throughout gestation, ewes had free access to water and were fed individually 1.4 kg/day of an alfalfa-based diet (consisting of 13.6% corn, 1.9% rice bran, 4.5% cottonseed meal, 3.0% cottonseed hull, 73.31% dehydrated alfalfa, 2.5% liquid binder, 0.4% soy oil, 0.5% ground limestone, 0.065% vitamin mixture, 0.15% salt mixture, and 0.075% mineral oil) that met the recommended NRC (National Research Council, 1985) requirements. The diet contained the following nutrients: 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, 1.23% calcium, 0.441% chloride, 0.285% magnesium, 0.306%

phosphorus, 2.01% potassium, 0.135% sodium, 0.221% sulfur, 0.385 ppm of cobalt, 7.67 ppm of copper, 0.258 ppm of iodine, 335 ppm of iron, 38.4 ppm of manganese, 0.555 ppm of selenium, 23.7 ppm of zinc, 147554 IU/kg of vitamin A, 316 IU/kg of vitamin D, 100 IU/kg of vitamin E, and 6.45 mg/kg of vitamin K. 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 at 24 h after the last feeding. All ewes were administered isofluorane (5%) to induce anesthesia and anesthesia was maintained with isofluorane (1%-5%). A mid-ventral laparotomy was then performed to expose the reproductive tract. Maternal uterine arterial blood (3 ml) and fetal umbilical venous blood (1 ml) were collected into heparinized tubes. Blood could not be obtained from fetal umbilical vein on Day 30 of gestation because of the small size of the vessels. Amniotic and allantoic fluids were obtained through the amniochorion and chorioallantoic membranes, respectively. Blood was centrifuged at 3000 x g at $4^{o}C$ for 10 min to obtain plasma. Plasma, amniotic and allantoic samples (0.5 ml) were deproteinized with 0.5 ml of 1.5 M $HClO_4$ and neutralized with 0.25 ml of 2 M K_2CO_3. Recovery of free amino acids from blood and fetal fluids was determined by adding known amounts of amino acid standards as previously described (Wu et al., 1998a) and was found to be greater than 95% for all amino acids.

Analysis of amino acids.

Amino acids, except for proline, were determined by fluorometric HPLC methods involving precolumn derivatization with *o*-phthaldialdehyde as previously described (Wu et al., 1997). The values for total cysteine in plasma and fluids represent free cysteine plus ½ cystine. Proline was measured using a fluorometric HPLC method involving precolumn derivatization with 9-

fluorenylmethyl chloroformate (Wu et al., 1997). Amino acids in samples were quantified on the basis of authentic standards (Sigma) using Millenium-32 Software (Waters, Milford, MA).

Calculations and statistical analysis

Amino acid concentrations in plasma, amniotic and allantoic fluids were calculated on the basis of the recovery rates of amino acids from ovine plasma, amniotic fluid, and allantoic fluid, respectively. For each gestational age, total content of amino acids in amniotic and allantoic fluids was calculated by multiplying amino acid concentrations by amniotic and allantoic fluid volumes, respectively. Concentrations of total α-amino acids (all measured amino acids except for β-alanine and taurine) were a mathematical sum of the individual α-amino acids. Data were subjected to least-squares analyses of variance, using the PROC GLM, PROC REG, and PROC CORR procedures of Statistical Analysis System (SAS, Cary, NC). Differences between means were determined by the Student-Newman-Keuls multiple-comparison test (SAS, Cary, NC). Statistical significance was set at a probability value of 0.05 or less.

Results

Fetal growth and fluid volume

Data regarding fetal weights as well as allantoic and amniotic fluid volumes are summarized in Table 3.1. Fetal weights increased with gestational age ($P < 0.01$, quadratic). The absolute growth rate of the fetal lamb was low (2.0 g/day) from Day 30 to Day 60 of gestation but increased rapidly (50.8 g/day) from Day 60 to Day 140. Weight gain from Day 120 to Day 140 of gestation was similar to that during the first 4 mo of gestation, indicating increased requirements for amino acids for protein deposition during late gestation. The volume of amniotic fluid increased progressively from Day 30 to Day 100 of gestation and then declined ($P < 0.01$, quadratic). The volume of allantoic fluid increased ($P < 0.01$) progressively from Day 40 to Day 120 of gestation and did

TABLE 3.1. Ovine fetal weight and volumes of allantoic and amniotic fluids

Day of gestation	Fetal weight (g)	Allantoic fluid (ml)	Amniotic fluid (ml)
30	0.88 ± 0.15 [g]	31 ± 4.0 [d]	2.2 ± 0.20 [e]
40	4.97 ± 0.30 [f]	11 ± 3.4 [e]	23.8 ± 2.5 [d]
60	61 ± 3.3 [e]	31 ± 3.8 [d]	190 ± 14 [c]
80	312 ± 14 [d]	46 ± 3.9 [c]	543 ± 26 [a]
100	1005 ± 65 [c]	199 ± 25 [b]	595 ± 43 [a]
120	2322 ± 80 [b]	491 ± 77 [a]	383 ± 58 [b]
140	4125 ± 418 [a]	366 ± 73 [a]	449 ± 71 [b]

[a-g]Data are means $\pm$ SEM for 4 ewes per gestational age. Means with different superscript letters within a column are different (P < 0.01).

not differ (P > 0.05) between Days 120 and 140 of gestation. Interestingly, the increase in allantoic fluid volume was closely correlated with the increase in fetal weight between Days 40 and 120 of gestation (r = 0.89, P < 0.01).

Amino acids in maternal uterine arterial and fetal umbilical venous plasma

Changes (P < 0.01) were observed in concentrations of all amino acids, except for proline and tyrosine, in maternal plasma during gestation (Table 3.2). Glycine was the most abundant amino acid in maternal plasma at all gestational ages (P < 0.01), accounting for approximately 25% of total α-amino acids. Alanine, glutamine, glycine, and serine were the most abundant amino acids in fetal umbilical venous plasma, which together contributed about 50% of total α-amino acids (Table 3.3). Marked changes (P < 0.01) were observed in concentrations of all amino acids, except for alanine, aspartate, isoleucine, leucine, phenylalanine, tryptophan, and valine, in fetal umbilical venous plasma between Days 40 and 140 of gestation. Fetal:maternal plasma ratios for amino

TABLE 3.2. Concentrations of amino acids (AA) in ovine uterine arterial plasma

Amino acid	Day of gestation							SEM
	30	40	60	80	100	120	140	
	μmol/L							
Ala	270 [a]	241 [ab]	216 [b]	273 [a]	307 [a]	201 [b]	205 [b]	20
β-Ala	19 [c]	27 [a]	22 [bc]	13 [d]	21 [bc]	25 [ab]	21 [bc]	1.6
Arg	191 [a]	104 [c]	118 [bc]	125 [bc]	134 [b]	189 [a]	131 [b]	7.5
Asn	38 [a]	22 [c]	27 [b]	28 [b]	36 [a]	27 [b]	37 [a]	2.1
Asp	17 [a]	9.8 [c]	6.8 [d]	7.5 [d]	13 [b]	12 [b]	8.1 [cd]	1.4
Cit	181 [bc]	158 [cd]	210 [a]	205 [a]	125 [e]	157 [cd]	141 [de]	10
Cys	188 [b]	209 [ab]	173 [b]	235 [a]	191 [b]	181 [b]	144 [c]	11
Gln	248 [bc]	177 [d]	215 [c]	264 [b]	370 [a]	203 [c]	350 [a]	15
Glu	137 [a]	88 [b]	48 [c]	91 [b]	109 [b]	90 [b]	61 [c]	9.3
Gly	566 [c]	587 [c]	484 [d]	748 [a]	676 [ab]	631 [bc]	655 [b]	35
His	70 [a]	56 [b]	53 [b]	56 [b]	55 [b]	41 [c]	44 [c]	2.6
Ile	66 [c]	58 [c]	61 [c]	65 [c]	71 [bc]	80 [ab]	85 [a]	5.0
Leu	111 [a]	99 [a]	76 [b]	101 [a]	97 [a]	108 [a]	104 [a]	8.7
Lys	119 [a]	90 [b]	72 [c]	92 [b]	83 [bc]	111 [a]	114 [a]	8.9
Met	22 [bc]	19 [c]	24 [b]	23 [b]	31 [a]	31 [a]	29 [a]	1.5
Orn	98 [a]	81 [b]	79 [b]	56 [c]	55 [c]	44 [d]	33 [e]	6.0
Phe	33 [bc]	31 [c]	24 [d]	125 [a]	36 [b]	39 [b]	33 [bc]	2.1
Pro	115	127	127	113	108	120	114	6.6
Ser	80 [b]	118 [a]	62 [c]	81 [b]	87 [b]	61 [c]	53 [c]	6.1
Taurine	54 [bc]	43 [c]	22 [d]	44 [c]	64 [b]	47 [c]	122 [a]	8.7
Thr	98 [a]	60 [b]	62 [b]	73 [b]	66 [b]	73 [b]	46 [c]	6.3
Trp	30 [a]	34 [a]	23 [b]	25 [b]	25 [b]	21 [b]	15 [c]	2.2
Tyr	43	52	39	45	48	45	41	3.2
Val	171 [a]	154 [ab]	148 [b]	122 [bc]	129 [bc]	128 [bc]	118 [c]	9.5
Total α-AA	2892 [a]	2573 [b]	2347 [b]	2954 [a]	2851 [a]	2593 [b]	2561 [b]	65

[a-e]Data are means with pooled SEM values for 4 ewes per gestational age. Means with different superscript letters within a row are different ($P < 0.01$).

TABLE 3.3. Concentrations of amino acids (AA) in ovine fetal umbilical venous plasma

Amino acid	Day of gestation						SEM
	40	60	80	100	120	140	
	μmol/L						
Ala	383	296	382	416	417	423	32
β-Ala	40 [e]	132 [c]	78 [d]	201 [b]	284 [a]	266 [a]	21
Arg	187 [d]	364 [b]	231 [c]	661 [a]	360 [b]	244 [c]	29
Asn	51 [d]	83 [b]	84 [b]	117 [a]	77 [c]	89 [b]	7.3
Asp	15	14	12	15	14	11	1.2
Cit	168 [d]	347 [ab]	216 [c]	226 [c]	302 [b]	393 [a]	29
Cys	190 [ab]	127 [c]	214 [a]	202 [a]	129 [c]	173 [b]	9.2
Gln	351 [d]	409 [d]	399 [d]	879 [a]	542 [c]	661 [b]	59
Glu	141 [a]	48 [b]	35 [c]	32 [c]	37 [c]	35 [c]	4.5
Gly	424 [d]	518 [cd]	612 [bc]	684 [b]	985 [a]	1041[a]	83
His	91 [a]	84 [a]	54 [b]	82 [a]	62 [b]	65 [b]	6.2
Ile	104	89	85	112	110	117	15
Leu	190	168	171	200	187	163	23
Lys	213 [ab]	272 [a]	199 [b]	204 [b]	181 [b]	133 [c]	21
Met	35 [d]	75 [b]	75 [b]	101 [a]	70 [b]	56 [c]	7.8
Orn	137 [b]	214 [a]	139 [b]	87 [c]	114 [bc]	122 [b]	17
Phe	97	110	123	117	107	109	8.3
Pro	209 [b]	206 [b]	208 [b]	203 [b]	303 [a]	319 [a]	19
Ser	290 [e]	379 [d]	441 [d]	670 [b]	903 [a]	554 [c]	47
Taurine	99 [b]	118 [b]	117 [b]	166 [a]	110 [b]	171 [a]	18
Thr	164 [c]	343 [a]	314 [a]	351 [a]	308 [ab]	266 [b]	27
Trp	37	56	48	52	46	40	3.6
Tyr	118 [b]	208 [a]	132 [b]	132 [b]	122 [b]	139 [b]	16
Val	378	271	235	307	276	311	31
Total α-AA	3973 [b]	4680 [b]	4410 [b]	5849 [a]	5651 [a]	5466 [a]	277

[a-e]Data are means with pooled SEM values for 4 ewes per gestational age. Means with different superscript letters within a row are different (P < 0.01).

TABLE 3.4. Ovine fetal:maternal ratios of plasma amino acids

Amino acid	Day of gestation						
	40	60	80	100	120	140	SEM
Ala	1.6 [b]	1.4 [b]	1.4 [b]	1.5 [b]	2.2 [a]	2.1 [a]	0.15
B-Ala	1.5 [d]	6.1 [c]	5.9 [c]	9.8 [b]	12 [a]	13 [a]	0.76
Arg	1.8 [c]	3.1 [b]	1.9 [c]	5.0 [a]	1.9 [c]	1.9 [c]	0.24
Asn	2.6	3.1	3.2	3.3	2.8	2.5	0.29
Asp	1.6 [b]	2.1 [a]	1.5 [b]	1.2 [b]	1.3 [b]	1.4 [b]	0.25
Cit	1.1 [c]	1.7 [b]	1.1 [c]	1.8 [b]	2.1 [b]	2.8 [a]	0.23
Cys	0.90 [b]	0.73 [c]	0.91 [b]	1.1 [a]	0.72 [c]	1.2 [a]	0.05
Gln	2.1 [bc]	1.9 [c]	1.5 [d]	2.4 [ab]	2.6 [a]	1.9 [c]	0.16
Glu	1.6 [a]	1.0 [b]	0.40 [d]	0.30 [d]	0.40 [d]	0.58 [c]	0.07
Gly	0.73 [c]	1.1 [b]	0.81 [c]	1.0 [b]	1.6 [a]	1.6 [a]	0.06
His	1.6 [a]	1.6 [a]	1.0 [b]	1.5 [a]	1.6 [a]	1.5 [a]	0.13
Ile	1.7	1.5	1.3	1.6	1.4	1.4	0.16
Leu	1.9	2.2	1.7	2.1	1.7	1.6	0.21
Lys	2.4 [b]	3.8 [a]	2.2 [b]	2.5 [b]	1.6 [c]	1.2 [c]	0.24
Met	2.1 [b]	3.2 [a]	3.3 [a]	3.3 [a]	2.2 [b]	2.0 [b]	0.31
Orn	1.7 [c]	2.7 [b]	2.5 [b]	1.6 [c]	2.6 [b]	3.7 [a]	0.24
Phe	3.1 [b]	4.6 [a]	1.0 [c]	3.3 [b]	2.8 [b]	3.3 [b]	0.27
Pro	1.7 [b]	1.7 [b]	1.9 [b]	2.0 [b]	2.7 [a]	2.9 [a]	0.16
Ser	2.5 [e]	6.1 [d]	5.6 [d]	7.8 [c]	15 [a]	11 [b]	0.89
Taurine	2.3 [b]	5.4 [a]	2.6 [b]	2.6 [b]	2.3 [b]	1.5 [c]	0.35
Thr	2.8 [c]	5.6 [a]	4.3 [b]	5.3 [a]	4.4 [b]	5.8 [a]	0.47
Trp	1.1 [c]	2.5 [a]	1.9 [b]	2.2 [ab]	2.2 [ab]	2.6 [a]	0.27
Tyr	2.3 [c]	5.4 [a]	3.0 [b]	2.7 [bc]	2.7 [bc]	3.3 [b]	0.35
Val	2.4	1.9	2.0	2.5	2.3	2.6	0.24

[a-e]Data are means with pooled SEM values for 4 ewes per gestational age.
Means with different superscript letters within a row are different (P < 0.01).

acids varied greatly, being the lowest for glutamate (e.g., 0.3 on Day 100 of gestation), between 1.5 and 3 for most amino acids throughout gestation, and greatest for serine (e.g., 15 on Day 120 of gestation) (Table 3.4). Interestingly, fetal:maternal plasma ratios for asparagine, isoleucine, leucine, and valine did not differ ($P > 0.05$) during gestation. In contrast, changes ($P < 0.01$) were observed in fetal:maternal plasma ratios for all other amino acids measured between Days 40 and 140 of gestation (Table 3.4).

Amino acids in amniotic fluid

Concentrations of amino acids in amniotic fluid are summarized in Table 3.5. As in fetal plasma, alanine, glutamine, glycine, and serine were the most abundant α-amino acids at all gestational ages, and contributed about 50% of total α-amino acids. Marked changes were observed in concentrations of all amino acids in amniotic fluid during pregnancy. Between Days 30 and 60 of gestation, concentrations of the following amino acids decreased ($P < 0.01$): glutamine, glycine, histidine, lysine, methionine, serine, and threonine. In contrast, concentrations of the following amino acids increased ($P < 0.01$) from Day 30 to Day 60 of gestation: arginine, aspartate, citrulline, cysteine, glutamate, leucine, ornithine, proline, taurine, tryptophan, and tyrosine. Except for β-alanine, aspartate, citrulline, cysteine, ornithine, serine, and taurine, the lowest concentrations of all amino acids were on Days 80-100 of gestation. Between Days 120 and 140 of gestation, concentrations of proline and threonine decreased ($P < 0.01$), whereas concentrations of the following amino acids increased ($P < 0.01$): β-alanine, arginine, citrulline, glutamine, glutamate, glycine, histidine, isoleucine, lysine, ornithine, and tryptophan.

Marked changes were observed in the total content of individual amino acids in amniotic fluid during gestation (Table 3.6). Between Days 30 and 60 of gestation, the total content of α-amino acids increased ($P < 0.01$) by approximately 80-fold.

TABLE 3.5. Concentrations of amino acids (AA) in ovine amniotic fluid

Amino acid	30	40	60	80	100	120	140	SEM
				µmol/L				
Ala	498 [b]	668 [a]	573 [b]	28 [e]	62 [d]	201 [c]	277 [c]	31
β-Ala	22 [d]	20 [d]	43 [c]	22 [d]	34 [cd]	256 [b]	396 [a]	10
Arg	120 [c]	105 [c]	177 [b]	17 [d]	15 [d]	155 [b]	212 [a]	14
Asn	74 [a]	65 [a]	82 [a]	12 [c]	16 [c]	36 [b]	41 [b]	4.6
Asp	6.8 [b]	2.8 [c]	10 [a]	5.1 [b]	13 [a]	14 [a]	12 [a]	1.7
Cit	42 [d]	61 [c]	207 [ab]	22 [e]	43 [d]	164 [b]	260 [a]	21
Cys	43 [e]	183 [a]	102 [c]	33 [e]	68 [d]	138 [b]	130 [b]	6.3
Gln	501 [a]	328 [c]	410 [b]	41 [f]	88 [e]	251 [d]	388 [bc]	25
Glu	27 [c]	41 [b]	67 [a]	8.7 [e]	18 [d]	28 [c]	41 [b]	3.7
Gly	426 [b]	230 [c]	281 [c]	48 [e]	94 [d]	436 [b]	579 [a]	38
His	82 [a]	48 [b]	56 [b]	4.4 [d]	17 [c]	23 [c]	43 [b]	4.1
Ile	31 [a]	39 [a]	37 [a]	3.4 [d]	6.1 [c]	24 [b]	36 [a]	3.8
Leu	75 [b]	97 [a]	109 [a]	24 [c]	7.1 [d]	87 [ab]	65 [b]	9.2
Lys	264 [a]	135 [c]	186 [b]	47 [e]	24 [f]	45 [e]	72 [d]	7.8
Met	58 [a]	44 [b]	43 [b]	1.6 [e]	8.3 [d]	21 [c]	27 [c]	2.5
Orn	63 [b]	38 [c]	122 [a]	85 [ab]	18 [d]	24 [d]	43 [c]	5.9
Phe	30 [a]	35 [a]	33 [a]	21 [b]	8.2 [c]	31 [a]	37 [a]	2.6
Pro	89 [c]	145 [b]	172 [a]	32 [d]	25 [d]	133 [b]	94 [c]	8.8
Ser	320 [b]	214 [cd]	186 [d]	84 [e]	275 [bc]	897 [a]	812 [a]	43
Taurine	31 [c]	44 [c]	89 [b]	84 [b]	299 [a]	105 [b]	93 [b]	9.4
Thr	231 [a]	82 [d]	103 [cd]	15 [f]	47 [e]	147 [b]	119 [c]	19
Trp	13 [b]	12 [b]	19 [a]	3.1 [e]	7.4 [d]	9.4 [c]	12 [b]	1.0
Tyr	33 [c]	49 [b]	45 [b]	3.2 [e]	10 [d]	52 [ab]	62 [a]	5.4
Val	150 [a]	148 [a]	138 [ab]	12 [d]	28 [c]	112 [b]	124 [ab]	15
Total α-AA	3177 [a]	2768 [b]	3158 [a]	550 [d]	898 [c]	3028 [a]	3487 [a]	153

[a-f] Data are means with pooled SEM values for 4 ewes per gestational age. Means with different superscript letters within a row are different (P < 0.01).

TABLE 3.6. Total content of amino acids (AA) in ovine amniotic fluid

Amino acid	Day of gestation							SEM
	30	40	60	80	100	120	140	
	μmol							
Ala	1.05 [e]	16 [d]	112 [a]	15 [d]	37 [c]	76 [b]	125 [a]	8.4
β-Ala	0.05 [f]	0.48 [e]	8.2 [d]	12 [d]	20 [c]	102 [b]	176 [a]	7.7
Arg	0.28 [f]	2.6 [e]	34 [c]	9.1 [d]	9.0 [d]	60 [b]	94 [a]	7.3
Asn	0.17 [f]	1.6 [e]	16 [ab]	6.6 [d]	9.5 [c]	14 [b]	19 [a]	1.2
Asp	0.02 [f]	0.07 [e]	1.9 [d]	2.8 [c]	7.6 [a]	5.2 [b]	5.3 [b]	0.1
Cit	0.09 [g]	1.5 [f]	39 [c]	12 [e]	26 [d]	63 [b]	116 [a]	11
Cys	0.09 [e]	4.4 [d]	19 [c]	17 [c]	42 [b]	51 [ab]	59 [a]	5.7
Gln	1.13 [f]	8.0 [e]	79 [b]	22 [d]	52 [c]	97 [b]	176 [a]	8.4
Glu	0.06 [e]	1.0 [d]	13 [b]	4.8 [c]	11 [b]	12 [b]	18 [a]	2.0
Gly	0.95 [f]	5.7 [e]	54 [c]	26 [d]	56 [c]	168 [b]	261 [a]	11
His	0.19 [e]	1.2 [d]	11 [b]	2.4 [c]	10 [b]	8.9 [b]	20 [a]	1.5
Ile	0.07 [g]	0.94 [f]	6.9 [c]	1.8 [e]	3.7 [d]	9.2 [b]	17 [a]	1.3
Leu	0.17 [f]	2.4 [e]	21 [b]	13 [c]	4.2 [d]	34 [a]	29 [a]	3.7
Lys	0.60 [e]	3.3 [d]	35 [a]	25 [b]	14 [c]	17 [c]	32 [a]	2.4
Met	0.13 [e]	1.1 [d]	8.3 [b]	0.9 [d]	4.9 [c]	8.1 [b]	12 [a]	1.6
Orn	0.14 [e]	0.91 [d]	23 [b]	46 [a]	11 [c]	9.3 [c]	20 [b]	3.4
Phe	0.07 [e]	0.90 [d]	6.4 [c]	11 [b]	4.8 [c]	12 [b]	17 [a]	1.1
Pro	0.20 [e]	3.5 [d]	33 [b]	18 [c]	15 [c]	52 [a]	43 [a]	3.9
Ser	0.71 [e]	5.2 [d]	36 [c]	47 [c]	163 [b]	344 [a]	366 [a]	24
Taurine	0.07 [e]	1.1 [d]	17 [c]	46 [b]	180 [a]	41 [b]	42 [b]	10
Thr	0.52 [e]	2.0 [d]	20 [b]	8.2 [c]	28 [b]	57 [a]	54 [a]	5.7
Trp	0.03 [f]	0.30 [e]	3.7 [c]	1.7 [d]	4.6 [b]	3.7 [c]	5.5 [a]	0.1
Tyr	0.07 [f]	1.2 [e]	8.7 [c]	1.8 [e]	5.9 [d]	20 [b]	29 [a]	0.9
Val	0.34 [f]	3.7 [e]	26 [c]	6.4 [e]	17 [d]	43 [b]	57 [a]	3.3
Total α-AA	7.1 [f]	68 [e]	607 [b]	299 [d]	537 [c]	1165 [b]	1576 [a]	121

[a-g] Data are means with pooled SEM values for 4 ewes per gestational age. Means with different superscript letters within a row are different (P < 0.01).

Between Days 60 and 80 of gestation, the total content of β-alanine, cysteine and serine did not differ (P > 0.05); the total content of aspartate, ornithine, phenylalanine, and taurine increased (P < 0.01); and the total content of other amino acids decreased (P < 0.01). Except for ornithine and taurine, the total content of all amino acids in amniotic fluid increased (P < 0.01) from Day 80 to Day 140 of gestation. The marked decrease in concentrations of total α-amino acids on Day 80 of gestation was inversely related to the increase in amniotic fluid volume (Table 3.1). However, the increases in concentrations of total α-amino acids in amniotic fluid during late gestation could not be accounted for by changes in amniotic fluid volume (Table 3.1).

Amino acids in allantoic fluid

Concentrations of amino acids in allantoic fluid are summarized in Table 3.7. Remarkable changes (P < 0.01) were observed in the concentrations of all amino acids in allantoic fluid during gestation. Concentrations of alanine, citrulline, and glutamine in allantoic fluid increased (P < 0.01) by 20-, 34-, and 18-fold, respectively, from Day 30 to Day 60 of gestation (Fig 3.1), and were approximately 80-, 30-, and 60-fold, respectively, those in ovine fetal plasma on Day 60 of gestation. Alanine, citrulline plus glutamine accounted for approximately 80% of total α-amino acid nitrogen in allantoic fluid during early pregnancy (Fig 3.2). Serine was the most abundant amino acid in allantoic fluid on Days 100 and 140 of gestation (P < 0.01), contributing 45% - 62% of total α-amino acid nitrogen (Fig 3.2). Concentrations of serine in allantoic fluid during late gestation were about 30-fold those in ovine fetal plasma (Table 3.3). The increase in concentrations of allantoic fluid serine was closely correlated with the increase in fetal weight (r = 0.69, P < 0.01).

Arginine was not among the most abundant amino acids in ovine allantoic fluid during early gestation (Table 3.7). However, arginine and citrulline were the second and third most abundant α-amino acids, respectively, in allantoic fluid on

TABLE 3.7. Concentrations of amino acids (AA) in ovine allantoic fluid

Amino acid	Day of gestation							SEM
	30	40	60	80	100	120	140	SEM
	μmol/L							
Ala	1199 [d]	16925 [b]	24714 [a]	3809 [c]	4277 [c]	927 [d]	791 [d]	542
β-Ala	94 [f]	462 [e]	2295 [b]	861 [d]	4190 [a]	1467 [c]	2717 [b]	158
Arg	336 [e]	639 [d]	821 [c]	887 [c]	2081 [a]	1220 [b]	1396 [b]	75
Asn	187 [e]	877 [b]	1904 [a]	304 [d]	610 [c]	154 [e]	217 [e]	58
Asp	47 [c]	220 [a]	206 [a]	115 [b]	194 [a]	128 [b]	180 [a]	19
Cit	277 [e]	2853 [b]	9682 [a]	1930 [c]	2178 [c]	1127 [d]	1216 [d]	206
Cys	145 [d]	167 [d]	402 [a]	427 [a]	340 [bc]	305 [c]	367 [b]	15
Gln	1217 [e]	12301 [b]	23502 [a]	3141 [d]	5001 [c]	814 [e]	1090 [e]	720
Glu	242 [c]	1175 [a]	712 [b]	112 [d]	230 [c]	118 [d]	313 [c]	66
Gly	1272 [c]	2449 [b]	3130 [b]	2527 [b]	5464 [a]	2220 [b]	1132 [c]	197
His	408 [d]	725 [b]	1501 [a]	238 [e]	537 [c]	249 [e]	310 [e]	46
Ile	112 [c]	190 [b]	183 [b]	108 [c]	316 [a]	69 [d]	88 [cd]	21
Leu	218 [d]	385 [bc]	454 [b]	573 [a]	94 [e]	236 [d]	345 [c]	23
Lys	544 [b]	1081 [a]	1014 [a]	1135 [a]	391 [c]	178 [d]	601 [b]	61
Met	70 [d]	69 [d]	118 [c]	62 [d]	386 [a]	95 [c]	233 [b]	17
Orn	117 [d]	471 [a]	495 [a]	360 [bc]	422 [ab]	103 [d]	343 [c]	34
Phe	107 [c]	64 [d]	121 [c]	652 [a]	269 [b]	93 [c]	107 [c]	22
Pro	211 [c]	522 [b]	573 [ab]	684 [a]	549 [b]	541 [b]	587 [ab]	32
Ser	510 [g]	1636 [f]	5039 [d]	3802 [e]	19072 [a]	11533 [c]	16468 [b]	480
Taurine	86 [d]	1110 [c]	2694 [b]	1239 [c]	4381 [a]	3015 [b]	2474 [b]	210
Thr	520 [d]	740 [c]	1067 [b]	716 [c]	1597 [a]	640 [c]	451 [d]	68
Trp	66 [c]	57 [c]	121 [b]	107 [b]	156 [a]	66 [c]	123 [b]	5.2
Tyr	176 [b]	168 [b]	258 [a]	56 [c]	303 [a]	138 [b]	148 [b]	21
Val	493 [b]	960 [a]	533 [b]	386 [c]	1021 [a]	258 [d]	311 [cd]	42
Total α-AA	8474 [d]	44675 [b]	76548 [a]	22130 [c]	45487 [b]	21213 [c]	26818 [c]	1103

[a-f] Data are means with pooled SEM values for 4 ewes per gestational age. Means with different superscript letters within a row are different (P < 0.01).
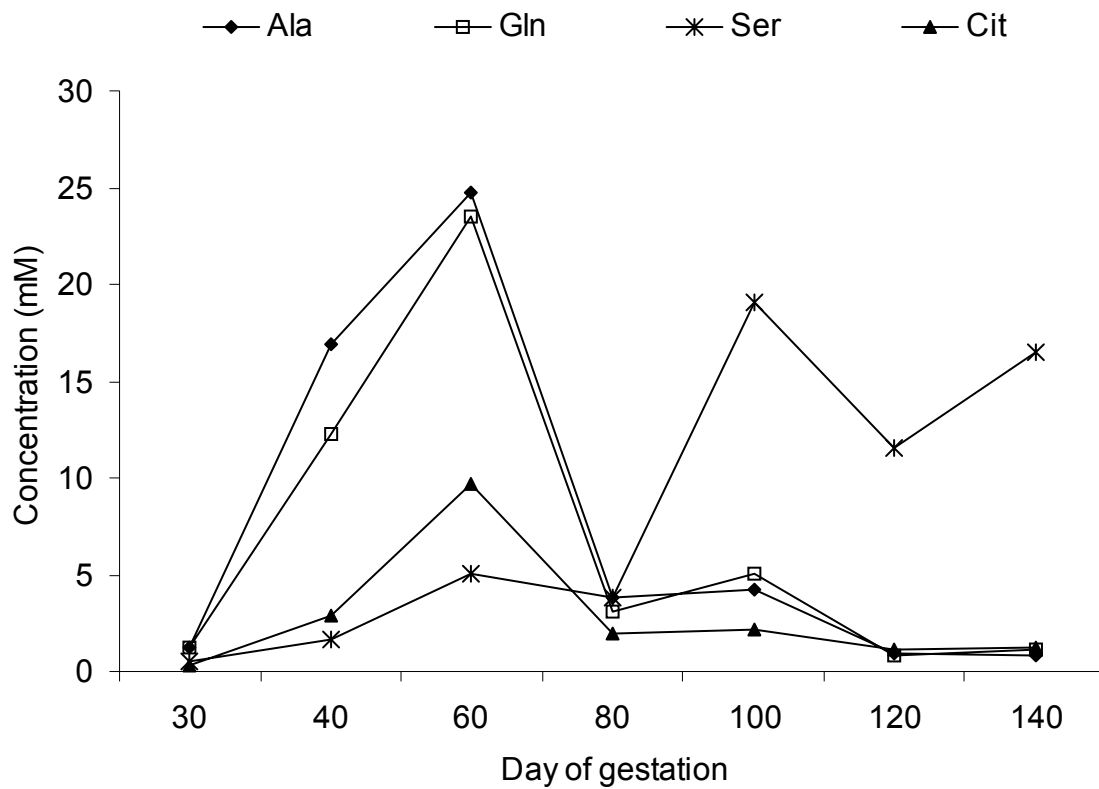
Fig 3.1   Concentrations of alanine, citrulline, glutamine, and serine in ovine allantoic fluid.   Pooled SEM values for alanine, citrulline, gltamine, and serine were 0.5, 0.2, 0.7, and 0.5 mM, respectively.
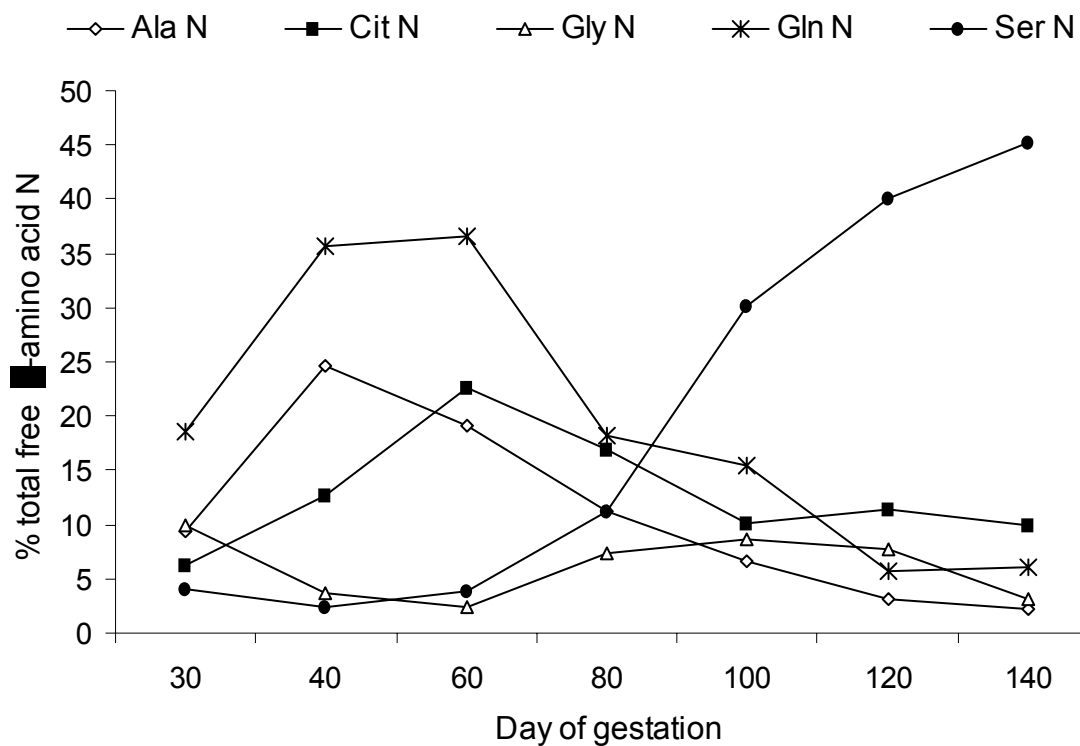
Fig 3.2 Percentage (%) total free α-amino acid nitrogen represented by alanine, citrulline, glycine, glutamine, and serine in ovine allantoic fluid. Total free α-amino acid N was calculated on the basis of nitrogen atoms of α- amino acids. Pooled SEM values for alanine, citrulline, glycine, glutamine, and serine were 3.2, 1.7, 3.5, 4.4, and 9.3 %, respectively.

Day 140 of gestation (P < 0.01), and contributed approximately 10% of total α-amino acids (Table 3.7). Between Days 100 and 140 of gestation, allantoic fluid was particularly rich in two β-amino acids: β-alanine (1.5-4.2 mM) and taurine (2.5-4.4 mM) (Table 3.7).

Except for arginine, cysteine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, tryptophan, tyrosine, and valine on Day 60 of gestation and for cysteine, methionine and phenylalanine on Day 40 of gestation, the total content of amino acids was much greater (P < 0.01) in allantoic fluid than in amniotic fluid at all gestational ages. As in amniotic fluid, marked changes were observed in the total content of individual amino acids in allantoic fluid during gestation (Table 3.8). Between Days 30 and 60 of gestation, the total content of amino acids, except for phenylalanine, tyrosine and valine, increased markedly (P < 0.01). Between Days 60 and 80 of gestation, the total content of arginine, cysteine, leucine, lysine, phenylalanine, and proline increased (P < 0.01), whereas the total content of alanine, β-alanine, asparagine, citrulline, glutamine, glutamate, histidine, taurine, and tyrosine decreased (P < 0.01). Except for leucine, the total content of all amino acids in allantoic fluid increased (P < 0.01) between Days 80 and 100 of gestation. Indeed, highest values were observed for alanine, glutamine, glycine, histidine, isoleucine, methionine, phenylalanine, threonine, tyrosine, and valine on Day 100 of gestation. Interestingly, total α-amino acid content in ovine allantoic fluid was fairly constant between Days 100 and 140 of gestation.

However, concentrations of protein in allantoic fluid were 1.3±0.5, 3.6±0.9, 9.8±1.3, 6.6±1.5, 2.7±0.7, 2.4±0.8, and 3.6±0.9 mg/ml, respectively, on Days 30, 40, 60, 80, 100, 120, and 140 of gestation. Concentrations of protein in amniotic fluid were 0.4±0.1, 0.2±0.1, 0.3±0.1, 0.4±0.1, 0.5±0.1, 1.3±0.5, and 1.3±0.4 mg/ml, respectively, on the same days.

TABLE 3.8. Total content of amino acids (AA) in ovine allantoic fluid

Amino acid	Day of gestation							
	30	40	60	80	100	120	140	SEM
				μmol				
Ala	38 [e]	186[d]	768 [a]	176 [d]	857 [a]	456 [b]	292 [c]	64
β-Ala	3.0 [g]	5.2 [f]	72 [d]	40 [e]	840 [b]	722 [c]	997 [a]	73
Arg	11 [e]	7.1 [e]	25 [d]	41 [c]	419 [b]	598 [a]	513 [a]	44
Asn	5.9 [f]	9.6 [e]	59 [c]	15 [d]	122 [a]	76 [b]	80 [b]	7.1
Asp	1.5 [d]	2.4 [d]	6.4 [c]	5.3 [c]	39 [b]	62 [a]	66 [a]	5.0
Cit	8.6 [f]	31 [e]	304 [c]	89 [d]	437 [b]	556 [a]	447 [b]	34
Cys	4.6 [e]	1.8 [f]	13 [d]	20 [c]	69 [b]	151 [a]	133 [a]	7.8
Gln	38 [e]	139 [d]	731 [b]	145 [d]	1004 [a]	399 [c]	402 [c]	88
Glu	7.6 [f]	13 [e]	23 [d]	5.2 [g]	46 [c]	58 [b]	116 [a]	10
Gly	40 [d]	27 [d]	98 [c]	115 [c]	1094[a]	1093 [a]	416 [b]	71
His	13 [c]	8.0 [d]	46 [b]	11 [d]	108 [a]	123 [a]	113 [a]	11
Ile	3.5 [d]	2.2 [d]	5.8[c]	5.0 [c]	64 [a]	34 [b]	32 [b]	4.2
Leu	6.7 [d]	4.3 [d]	15 [c]	26 [b]	19 [c]	117 [a]	128 [a]	11
Lys	17 [e]	12 [e]	32 [d]	52 [c]	79 [b]	87 [b]	221 [a]	19
Met	2.1 [d]	0.75[e]	3.7[c]	2.8 [c]	77 [a]	48 [b]	85 [a]	6.8
Orn	3.6 [e]	5.2 [e]	16 [d]	17 [d]	84 [b]	51 [c]	126 [a]	15
Phe	3.4 [d]	0.71[e]	3.8[d]	30 [c]	54 [a]	45 [b]	40 [b]	3.7
Pro	6.6 [e]	5.7 [e]	18 [d]	32 [c]	112 [b]	267 [a]	215 [a]	18
Ser	16 [d]	18 [d]	159 [c]	176 [c]	3816 [b]	5665 [a]	6029 [a]	423
Taurine	2.7 [g]	12 [f]	85 [d]	57 [e]	877 [c]	1491 [a]	907 [b]	95
Thr	17 [d]	8.1 [e]	34 [c]	33 [c]	320 [a]	316 [a]	166 [b]	16
Trp	2.1 [d]	0.63[e]	3.8[c]	4.9 [c]	32 [b]	33 [b]	45 [a]	4.5
Tyr	5.8 [c]	1.9 [d]	8.0[c]	2.5 [d]	61 [ab]	68 [a]	55 [b]	3.9
Val	16 [c]	11 [d]	17 [c]	18 [c]	204 [a]	127 [b]	115 [b]	11
Total α-AA	268 [e]	496 [d]	2392 [b]	1020 [c]	9116 [a]	10432 [a]	9836 [a]	1237

[a-g] Data are means with pooled SEM values for 4 ewes per gestational age.
Means with different superscript letters within a row are different (P < 0.01).

Discussion

The sheep is a widely used animal model for studying human fetal-placental development (Lemons et al., 1976; Meier et al., 1981; Rice et al., 1987; Marconi et al., 1989; Moores et al., 1994; Stewart et al., 2000; Battaglia and Regnault, 2001; Osgerby et al., 2002). However, little is known about the dynamics of change in concentrations of amino acids in ovine amniotic and allantoic fluids. To our knowledge, this is the first report of developmental changes in concentrations of amino acids in ovine maternal arterial plasma, fetal umbilical venous plasma, and fetal fluids. Three unique, major findings emerged from this study: 1) ovine fetal:maternal plasma ratios for amino acids changed greatly during gestation, 2) the marked changes in concentrations of amino acids in ovine allantoic and amniotic fluids were associated with conceptus development, and 3) alanine, citrulline, and serine were unusually abundant in ovine allantoic fluid compared with any other biological fluid in animals (for example, see Lemons et al., 1976; Marconi et al., 1989; Snell and Fell, 1990; Curthoys and Watford, 1995; Wu et al., 1996a; Wu and Morris, 1998; Stipanuk and Watford, 2000).

To our knowledge, this is also the first report of ovine fetal:maternal plasma ratios for amino acids throughout the entirety of gestation. Fetal:maternal plasma ratios for glutamate and serine were remarkably low and high, respectively, during late gestation (Table 3.4). *In vivo* studies have demonstrated that there is little uterine uptake of glutamate from maternal plasma, but that there is placental uptake of glutamate (derived largely from glutamine hydrolysis in fetal liver) occurs in the ovine fetus (Marconi et al., 1989). Thus, extensive placental catabolism of glutamate (Battaglia and Regnault, 2001) and a high rate of fetal utilization of glutamate (a major amino acid in tissue protein) (Meier et al., 1981) likely are the major factors responsible for its low concentrations in fetal plasma compared with maternal plasma. In contrast, uterine uptake of serine from maternal plasma occurs, but little transplacental transport of serine to the ovine fetus takes place because of its

catabolism by uteroplacental tissues (Moores et al., 1994; Battaglia and Regnault, 2001). Thus, large amounts of serine are synthesized from glycine and N^5,N^{10}-methylenetetrahydrofolate via serine hydroxymethyltransferase and from 3-phosphoglycerate (an intermediate of glycolysis) and glutamate via phosphoglycerate dehydrogenase and phosphoserine aminotransferase (Marconi et al., 1989; Moores et al., 1994). All these enzymes are present in fetal ovine liver and kidney, with the liver being the major organ for serine synthesis in fetal sheep (Narkewicz et al., 1999, 2002).

Amniotic fluid is composed of water and electrolytes from both the fetus (kidneys, lungs, epidermis, and fetal blood vessels in the placenta and umbilical cord) and the mother (decidual blood vessels via amniotic membranes) (Schmidt, 1992). This fluid is removed by both the fetus and the mother through the same channels, along with the participation of the fetal intestine after swallowing (Schmidt, 1992). Thus, with the development of intestinal amino acid transport systems during gestation (Sagawa et al., 1979), the drinking of amniotic fluid provides a source of amino acids for fetal utilization. The nutritional significance of amniotic fluid is graphically illustrated by the finding that esophageal ligation, which prevents entry of this fluid into the small intestine, results in intrauterine growth retardation in fetal sheep (Trahair and Harding, 1995). Glutamine, a major fuel for enterocytes (Stipanuk and Watford, 2000) and an abundant amino acid in amniotic fluid (Table 3.5), may be an important nutrient in this fluid that stimulates intestinal growth and development.

Allantoic fluid is derived from fetal and maternal secretions but primarily from placental transport mechanisms (Bazer, 1989). Although early anatomical studies suggested that the allantoic sac served as a reservoir for fetal wastes, it is now clear that allantoic fluid nutrients may be absorbed by the allantoic epithelium into the fetal-placental circulation and utilized by fetal-placental tissues (Bazer, 1989). The increases in concentrations of total amino acids in allantoic fluids during early pregnancy could not be accounted for by changes in allantoic fluid volume (Table 3.1) or by changes in concentrations of amino acids

in maternal and fetal plasma (Tables 3.2 and 3.3). Interestingly, between Days 30 and 60 of gestation, Na^+ and Cl^- concentrations in ovine allantoic fluid decrease by 40 and 39 mEq/L, respectively, with a combined decrease of 79 mEq/L (Bazer, 1989). Such a decrease in electrolyte concentrations is closely matched by an increase of 73 mmol/L amino acids in allantoic fluid (Table 3.7), suggesting an important role for amino acids as regulators of osmolality in allantoic fluid.

Ovine allantoic fluid was particularly rich in four of the traditionally classified nonessential amino acids: alanine, citrulline, glutamine, and serine (Table 3.7). In contrast to alanine, glutamine and serine, citrulline (a nonprotein amino acid) is not a building block for tissue protein synthesis and is virtually absent from the diet. Citrulline was the third most abundant amino acid in ovine allantoic fluid on Day 60 of gestation (Table 3.7). Although glutamine concentrations may be as high as 20 mM in human skeletal muscle (Curthoys and Watford, 1995), the unusual abundance of alanine and citrulline in ovine allantoic fluid on Day 60 of gestation and of serine during late gestation has not been reported for any other biological fluid in animals (e.g., Lemons et al., 1976; Marconi et al., 1989; Snell and Fell, 1990; Curthoys and Watford, 1995; Wu et al., 1996; Wu and Morris, 1998; Stipanuk and Watford, 2000). For comparison, concentrations of alanine, glutamine, citrulline, and serine in porcine allantoic fluid were 0.28, 3.4, 0.07, and 0.90 mM, respectively, on Day 40 of gestation (Wu et al., 1996), and 0.67, 0.70, 0.03, and 1.2 mM, respectively, on Day 110 of gestation (Wu et al., 1995).

Our results raise important questions regarding the origin and function of alanine, glutamine, citrulline, and serine in ovine fetal-placental nutrition and metabolism. Both glutamine and alanine may be synthesized from branched-chain amino acids in fetal ovine skeletal muscle (Goodwin et al., 1987). On the basis of maternal arterial-fetal umbilical venous differences in amino acid concentrations (Tables 3.2 and 3.3), we suggest that the ovine placenta synthesizes citrulline. Indeed, our *in vitro* studies demonstrated the formation of

citrulline from glutamine in incubated ovine placenta (data not shown) as previously reported for the porcine small intestine (Wu et al., 1994a). As noted above, serine is synthesized from glycine and from 3-phosphoglycerate (an intermediate of glycolysis) and glutamate (a metabolite of glutamine) in the liver and kidneys of fetal sheep (Narkewicz et al., 1999, 2002). Alanine, glutamine, and serine are major glucogenic precursors in humans (Stipanuk and Watford, 2000) and ewes (Clark et al., 1976). Serine also plays an important role in one-carbon unit metabolism essential for 2'-deoxythymidylate synthesis and methylation (Snell and Fell, 1990). Glutamine is a major fuel for the fetus (Bell et al., 1989) and is essential for the synthesis of nucleotides, $NAD(P)^+$, and aminosugars (glucosamine-6-phosphate, UDP-N-acetylgalactosamine, and UDP-N-acetylglucosamine, a precursor for the formation of all macromolecules containing amino sugars) (Stipanuk and Watford, 2000; Flynn et al., 2002). In addition, serine participates in the synthesis of phosphatidylserine and ceramide (signaling molecules) (Stipanuk and Watford, 2000). All of these events are critical for DNA synthesis and, thus, cell proliferation.

In contrast to high concentrations of arginine (4-6 mM) (Fig 3.3) in porcine allantoic fluid during early gestation (Wu et al., 1995a, 1996a, 1998a), arginine was not a major amino acid in ovine allantoic fluid during early gestation (Table 3.7). Concentrations of arginine in ovine allantoic fluid on Days 60, 100, and 140 of gestation were only 16%, 11%, and 8.5%, respectively, of those of serine (Table 3.7). Thus, our findings do not support the hypothesis that arginine is the most abundant amino acid in ovine allantoic fluid. Why is citrulline, but not arginine, particularly abundant in ovine allantoic fluid during early gestation? Several answers to this intriguing question are possible. First, arginase activity is present in ovine allantoic fluid on Days 30 and 60 of gestation but is not detectable in porcine allantoic fluid on Days 30 to 60 of gestation (unpublished data). The presence of arginase in ovine allantoic fluid would hydrolyse arginine and reduce its availability to the fetus. Because citrulline is an effective
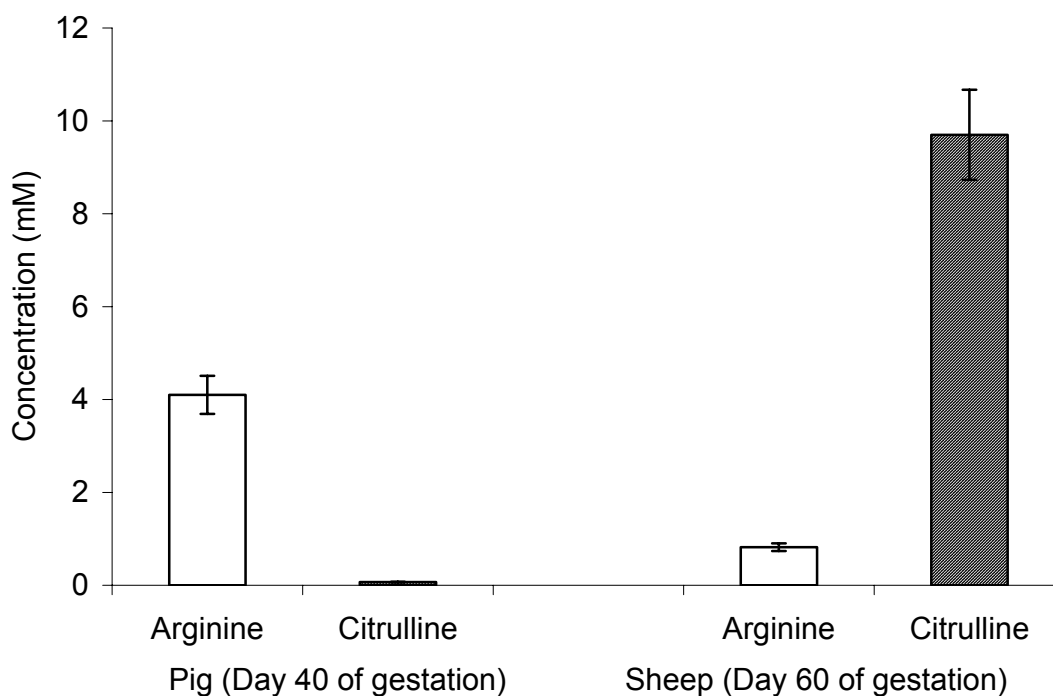
Fig 3.3 Concentrations of arginine and citrulline in porcine and ovine allantoic fluids during early gestation.

precursor for arginine synthesis in all animals because of the widespread presence of argininosuccinate synthase and lyase in animal tissues (Wu and Morris, 1998), high concentrations of citrulline in ovine allantoic fluid would serve as an efficient reservoir of precursor for arginine in the fetus. Second, citrulline is a neutral amino acid. Thus, unlike arginine (a basic amino acid), citrulline does not disturb the acid-base balance in ovine allantoic fluid, even at high concentrations. Third, citrulline is an efficient antioxidant, protecting DNA, lipids and proteins from hydroxyl radical-induced oxidative damage (Akashi et al., 2001). This effect of citrulline may contribute to a protective environment for fetal growth and development in sheep.

Collectively, our findings indicate that different strategies are used by different animal species to conserve arginine, the most abundant nitrogen carrier in tissue proteins (Meier et al., 1981; Wu et al., 1999a). Whatever the

differences among species, the unusual abundance of either citrulline (an effective precursor of arginine) in ovine allantoic fluid (Table 3.7) or arginine in porcine allantoic fluid (Wu et al., 1995a, 1996a, 1998a) during early gestation raises intriguing and important questions regarding the physiological significance of arginine-dependent pathways in fetal-placental nutrition and development. In this regard, it is noteworthy that maternal undernutrition decreases arginine concentrations in porcine fetal plasma and allantoic fluid (Wu et al., 1998a) and impairs fetal growth (Wu et al., 1998a; Osgerby et al., 2002), and may also program permanent structural, metabolic and functional alterations (Barker and Clark, 1997; Symonds et al., 2001). We recently determined that maternal under-nutrition in sheep (50% of NRC nutrient requirements) from Day 28 to Day 78 of gestation decreased concentrations of citrulline, serine, glutamine, and alanine in fetal plasma and allantoic fluid by 35 - 45% and retarded fetal growth by 32% on Day 78 of gestation (unpublished data). Because recent epidemiological studies in humans suggest links between intrauterine growth retardation and development of chronic disease (e.g., diabetes, hypertension, and coronary heart disease) later in life (Barker and Clark, 1997; Symonds et al., 2001), our novel results may have important implications for both intrauterine growth retardation and fetal origins of diseases in adults.

In conclusion, remarkable changes occur in concentrations of amino acids in ovine fetal allantoic fluid between Days 30 and 140 of gestation. In this fluid, alanine, citrulline, plus glutamine contributed approximately 80% of total α-amino acids during early gestation, and serine accounted for approximately 60% of total α-amino acids during late gestation. These novel findings raise important questions regarding placental and fetal metabolism of the traditionally classified nonessential amino acids, and provide a new database for further studies to define their roles in ovine conceptus development.

CHAPTER IV
DEVELOPMENTAL CHANGES IN POLYAMINE LEVELS
AND SYNTHESIS IN OVINE CONCEPTUS

Introduction

The placentae of all mammalian species undergo rapid formation of new blood vessels (angiogenesis) and marked growth during pregnancy (Ford, 1995; Reynolds and Redmer, 2001). Placental angiogenesis is necessary to increase placental-fetal blood flow and the supply of nutrients for transfer from maternal to fetal blood. Therefore, placental growth is a critical factor for controlling the survival, growth, and development of the fetus, and a better understanding of factors that regulate placental growth is essential to improve the 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) (Alexander, 1964; Reynolds and Redmer, 1995). The ovine placenta has 60 to 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 (Alexander, 1964).

Polyamines (putrescine, spermidine, and spermine) are key regulators of angiogenesis, early mammalian embryogenesis, placental trophoblast growth, and embryonic development in the uterus (Fozard et al., 1980; Henningsson et al., 1982). Through binding intracellular RNA, DNA, nucleotide triphosphates, proteins, and other negatively charged molecules, polyamines (polycationic molecules) regulate gene expression, signal transduction, ion channel function, as well as DNA and protein synthesis (Igarashi and Kashiwagi, 2000).

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Polyamines are also endogenous scavengers of reactive oxygen species, thereby protecting DNA, proteins, and lipids from oxidative damage (Chattopadhray et al., 2003). Thus, polyamines are essential for cell proliferation, differentiation, and function.

Ornithine decarboxylase (ODC) is a rate-controlling enzyme of the polyamine-synthetic pathway in mammalian cells (Tabor and Tabor, 1984). It converts ornithine (a product of arginine hydrolysis by arginase) into putrescine, which is subsequently converted into spermidine and spermine by spermidine and spermine synthases, respectively. Despite previous studies of the role of polyamines in placental and fetal development (Fozard et al., 1980; Henningsson et al., 1982), little is known about changes in placental polyamine synthesis associated with conceptus development in any species. Although placental ODC activity has been reported for some mammals, including rats, mice and pigs (Forzard et al., 1980; Henningsson et al., 1982; Wu et al., 1998b), it is necessary to determine rates of polyamine synthesis in intact tissues or cells. This is because a change in enzyme (e.g., ODC) activity measured under *in vitro* assay conditions does not necessarily indicate a change in metabolic flux or product formation (Fell, 1997; Wu et al., 2000c). Information about polyamine synthesis 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.

We recently reported marked increases in concentrations of both ornithine and arginine (substrates for polyamine synthesis) in ovine allantoic fluids (a reservoir for nutrients in the fetus) between Days 30 and 60 of gestation (Kwon et al., 2003a). Such changes coincide with the period of most rapid growth of the ovine placenta (Alexander, 1964; Reynolds and Redmer, 1995). On the basis of this observation, we hypothesized that placental ODC and arginase activities and polyamine synthesis were maximal during the first half of pregnancy. This hypothesis was tested using ewes between Day 30 and Day 140 of gestation. Endometrium was analyzed in the same manner, because it is

closely associated with placental development and function (Wimsatt, 1950; Stewart et al., 2000; Gray et al., 2001). Because amniotic and allantoic compartments are integral parts of the ovine conceptus essential for fetal growth (Bazer, 1989), we also determined changes in concentrations of polyamines in amniotic and allantoic fluids during pregnancy.

Materials and Methods

Chemicals

Putrescine, spermidine, spermine, and amino acids were purchased from Sigma Chemicals (St. Louis, MO). L-[U-^{14}C]ornithine and L-[U-^{14}C]arginine were obtained from American Radiolabeled Chemicals (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade water and methanol were purchased from Fisher Scientific (Fair Lawn, NJ).

Experimental animals

Columbia cross-bred ewes were mated to Suffolk rams when detected in estrus (Day 0) and at 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 as well as amniotic and allantoic fluids. Because there were marked changes in physiological parameters in the ovine conceptus during pregnancy (e.g., Gray et al., 2001; Kwon et al., 2003a) and because coefficients of variations for all measured parameters were relatively small (< 3-7%), we used 4 ewes per day of gestation on the basis of statistical power calculation. In preliminary studies, we determined that concentrations of polyamines in placental and endometrial tissues, amniotic fluid, or allantoic fluid were not affected by the number of fetuses (data not shown). Thus, samples were obtained from one randomly selected fetus per ewe for polyamine analysis when there were twin fetal lambs. None of the ewes in the study had more than twin 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 NRC (National Research Council, 1985) requirements (Kwon et al., 2003a). 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 8:00 and 9:00 A.M., 24 h after the last feeding (Kwon et al., 2003a). All ewes were administered isofluorane (5%) via an inhalation mask to induce anesthesia which was maintained with isofluorane (1-5%). A mid-ventral laparotomy was performed to expose the reproductive tract. Amniotic and allantoic fluids were collected through amniochorion and chorio-allantoic membranes, respectively. Placentomes, intercotyledonary placenta and intercaruncular endometrium were obtained on all days of gestation. A portion of these tissues was used immediately for ODC assays and metabolic studies, and the remaining tissues were stored at $-80\ ^{\circ}C$ for determination of polyamines, amino acids and arginase activity within 1 week. 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 ODC activity

ODC activity in placental and endometrial tissues was measured using L-[1-^{14}C]ornithine as we described for porcine tissues (Wu et al., 1998b). Briefly, tissues (~200 mg) were homogenized, using a glass homogenizer, in 0.5 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 0.2 mM pyridoxal-5-phosphate, 1 mM EDTA, 2.5 mM dithiothreitol, 150 mM sucrose, and protease inhibitors (5 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg /ml

chymostatin, and 5 μg /ml pepstatin A). The homogenizer was rinsed with 0.5 ml of the buffer, and the combined homogenates were centrifuged at 13,000 g for 15 min at 4°C. The supernatants (free of mitochondria) were used for ODC assays to prevent the potential production of $^{14}CO_2$ from [1-^{14}C]ornithine via mitochondrial ornithine aminotransferase and Krebs cycle enzymes (Wu et al., 1996b, 2000c). Pyrroline-5-carboxylate reductase activity was measured as a marker for cytosolic enzymes as previously described (Wu et al., 1996b). The assay mixture (0.5 ml) for ODC consisted of 0.2 mM L-[1-^{14}C]ornithine (5500 dpm/nmol), 0.2 mM pyridoxal-5-phosphate, 0.2 mM EDTA, 0.5 mM dithiothreitol, enzyme preparations (equivalent to ~20 mg tissue), and 50 mM sodium phosphate buffer (pH 7.2). On Days 40 and 140 of gestation, ODC activity was also measured in the presence of 2 mM ornithine. Radioactivity blanks containing [1-^{14}C]ornithine but no enzyme preparations were run. After incubation at 37°C for 1 h, $^{14}CO_2$ was collected in 0.2 ml NCS (a trademark name; Amersham, Arlington Heights, IL), and its radioactivity was measured in a liquid scintillation counter (Packard, Meriden, CT). Recovery of $^{14}CO_2$ from the incubation medium was 96%, as determined using a known amount of [^{14}C]NaHCO$_3$. Rates of ornithine decarboxylation by ODC were calculated by dividing the radioactivity of collected $^{14}CO_2$ (dpm) by the specific activity of [1-^{14}C]ornithine (dpm/nmol) in the assay solution. Enzyme activity was expressed on the basis of the amount of tissue in the assay mixture. Coefficients of variation for intra- and inter-assays of ODC were < 4% and 7%, respectively.

Determination of arginase activity

Arginase activity in placental and endometrial tissues was measured by quantifying ornithine production from arginine as we described previously for porcine tissues (Wu et al., 1996b). Briefly, placental or endometrial tissues (~200 mg) were homogenized, using a glass homogenizer, in 1 ml of homogenization buffer (pH 7.4) containing 300 mM sucrose, 1 mM EDTA, 3 mM dithiothreitol, 5 mM HEPES, protease inhibitors (5 μg/ml phenylmethylsulfonyl

fluoride, 5 μg/ml aprotinin, 5 μg /ml chymostatin, and 5 μg /ml pepstatin A), and 0.5% Triton X-100. The homogenizer was rinsed with 1 ml of the above buffer, and the combined homogenates were used for arginase assays. The assay mixture (1 ml), which consisted of 50 mM potassium phosphate buffer (pH 7.5), 10 mM arginine, 10 mM $MnCl_2$ and tissue homogenates (equivalent to ~ 10 mg tissue), was incubated at 37^oC for 0 or 15 min. On Days 40 and 140 of gestation, arginase activity was also measured in the presence of 2 mM arginine. The reaction was terminated by addition of 0.2 ml of 1.5 M $HClO_4$, and ornithine produced was analyzed by HPLC (Wu et al., 1998b). Rates of arginine hydrolysis by arginase were calculated on the basis of ornithine production during a 15 min assay period. Enzyme activity was expressed on the basis of the amount of tissue in the assay mixture. Coefficients of variation for intra- and inter-assays of arginase were < 3% and 5%, respectively.

Fetal fluid samples were centrifuged at 3000 $\times$ g at 4°C for 10 min. Supernatant fractions were assayed for arginase activity and arginine concentrations (1mM and 10mM) by monitoring the conversion of L-[guanido-^{14}C]arginine to [^{14}C]urea during 20 min incubation. For example, the assay mixture (1 ml), which consisted of 150 mM potassium phosphate buffer (pH 7.5), 10 mM arginine, 10 mM $MnCl_2$ and fluid samples, was incubated at 37^oC for 20 min. The reaction was terminated by addition of 0.2 ml of 1.5 M $HClO_4$.

Determination of polyamines and amino acids

Placental and endometrial tissues (~200 mg) were homogenized at 4°C in 1 ml of 1.5 mM $HClO_4$ using a glass homogenizer, and the homogenizer was rinsed with 1 ml of 1.5 mM $HClO_4$. The combined homogenates were transferred to 12 $\times$ 75 mm polypropylene tubes and neutralized with 1 ml of 2 mM K_2CO_3. The homogenates were centrifuged at 3000 $\times$ g and 4°C for 15 min to obtain the supernatant fluid for polyamine analysis. Fetal fluid samples were centrifuged at 3000 x g at 4^oC for 10 min. An aliquot of the supernatant (0.5 ml) was deproteinized with 0.5 ml of 1.5 M $HClO_4$, followed by neutralization with

0.25 ml of 2 M K_2CO_3. Recovery of putrescine, spermidine, spermine, ornithine, and arginine from placental and endometrial samples as well as fetal fluids was determined by adding known amounts of polyamine and amino acid standards (Wu et al., 2000c), and was found to be > 93% for polyamines and > 96% for amino acids.

Polyamines were analyzed by an ion-pairing HPLC method involving precolumn-derivatization with o-phthaldialdehyde (Wu et al., 2000c). Briefly, 100 μl of sample was mixed with 100 μl of 1.2% benzoic acid (in 40 mM sodium borate, pH 9.5) and 1.4 ml HPLC-grade H_2O. An aliquot (100 μl) of the assay mixture was derivatized, in an autosampler (Model 712 WISP, Waters Inc., Milford, MA), with 100 μl of 30 mM o-phthaldialdehyde (in 3.1% Brij-35, 50 mM 2-mercaptoethanol and 40 mM sodium borate, pH 9.5).   An aliquot of the derivatized mixture (25 μl) was injected into a Supelco 3-μm reversed-phase C_{18} column (150 x 4.6 mm I.D.; Sigma Chemicals, St. Louis, MO) guarded by a Supelco 40-μm reversed-phase C_{18} column (50 x 4.6 mm I.D.).   Polyamines were separated using a solvent gradient consisting of solution A (0.1 M sodium acetate, 2 mM sodium dodecyl sulfate, 0.5% tetrahydrofuran and 9% methanol, pH 7.2) and solution B (100% methanol and 2 mM sodium dodecyl sulfate). Putrescine, spermidine and spermine in samples were quantified on the basis of authentic standards, using Millenium-32 Software (Waters, Milford, MA).

Amino acids were analyzed using the HLPC system as described above, except that the mobile phase solutions did not contain the ion-pairing agent sodium dodecyl sulfate (Wu et al., 1996b).   Amino acids in samples were quantified on the basis of authentic standards, using Millenium-32 Software (Waters, Milford, MA) (Wu et al., 2000c).

Determination of polyamine synthesis

Polyamine synthesis was determined in placental and endometrial tissues using L-[U-^{14}C]arginine and L-[U-^{14}C]ornithine, as we described for porcine tissues (Wu et al., 2000c). Briefly, placental or endometrial tissues (~500 mg)

were rinsed twice with oxygenated (95% O_2/5% CO_2) Basal Medium Eagle (BME) (GIBCOBRL, Grand Island, N.Y.), and then incubated at 37 ^{o}C for 3 h in 2 ml of oxygenated (95% O_2/5% CO_2) BME containing 5 mM glucose, 0.5 mM L-methionine, and either 1 mM L-arginine plus 2 μCi L-[U-^{14}C]arginine (1 μCi/μmol) or 1 mM L-ornithine plus 2 μCi L-[U-^{14}C]ornithine (1 μCi/μmol). Incubations, in which medium contained all of the above components but no tissues, were run as blanks. The ^{14}C-labeled substrates were used to improve the sensitivity of detecting polyamine synthesis in placental and endometrial tissues. Incubations were terminated by addition of 0.2 ml of 1.5 M $HClO_4$. The acidified tissues plus medium were homogenized using a glass homogenizer, and the homogenates were neutralized with 0.1 ml of 2 M K_2CO_3. The neutralized extracts were dried in a Model RC10.10 centrifugal evaporator (Jouan Inc., Winchester, VA), and suspended in 0.3 ml of H_2O for separation of [^{14}C]putrescine, [^{14}C]spermidine and [^{14}C]spermine by HPLC. The fractions containing [^{14}C]putrescine, [^{14}C]spermidine and [^{14}C]spermine were collected from the HPLC column for measuring radioactivities in a Packard liquid scintillation counter.   Recovery of [^{14}C]putrescine, [^{14}C]spermidine and [^{14}C]spermine from the HPLC column was determined using known amounts of [^{14}C]polyamine standards, and was found to be greater than 91%. Blank radioactivities were subtracted from sample values. Rates of production of putrescine, spermidine and spermine were calculated on the basis of intracellular specific activities of [^{14}C]ornithine, which were measured as described by Wu (1997).

Calculations and statistical analysis

Concentrations of polyamines and amino acids in placental and endometrial tissues as well as fetal fluids were calculated on the basis of the recovery rates of putrescine, spermidine, spermine, ornithine and arginine from the ovine placental and uterine tissues and fetal fluids. For each gestational age, total content of polyamines in amniotic and allantoic fluids was calculated

by multiplying polyamine concentrations by amniotic and allantoic fluid volumes, respectively. Concentrations of total polyamines were a mathematical sum of putrescine, spermidine and spermine. For each gestational age, total activity of arginase in amniotic and allantoic fluids was calculated by multiplying arginase activity by amniotic and allantoic fluid volumes, respectively. Data obtained between Days 30 and 140 of gestation were subjected to least squares analyses of variance and one-way analysis of variance (Steel et al., 1997), using the PROC GLM, PROC REG, and PROC CORR procedures of Statistical Analysis System (SAS, Cary, NC). Differences between means were determined by the Student-Newman-Keuls multiple comparison test following one-way analysis of variance (Steel et al., 1997). Differences between ODC and arginase activities in the same tissue on Day 40 or 140 of gestation were determined by paired t-test. Statistical significance was set at a probability value of ≤ 0.05.

Results

ODC activity in placental and endometrial tissues

As summarized in Table 4.1, marked changes ($P < 0.01$) in ODC activity occurred in ovine placental and endometrial tissues during conceptus development. In intercotyledonary placenta, ODC activity was the highest on Days 30 to 40 of gestation and then decreased ($P < 0.01$) on Day 60 of gestation. No change in intercotyledonary placental ODC activity was observed between Days 60 and 140 of gestation. In intercaruncular endometrium, ODC activity increased ($P < 0.01$) by 35% between Days 30 and 40 of gestation, declined ($P < 0.01$) progressively between Days 40 and 100, and remained at low values on Days 100 to 140. Placentomes exhibited the greatest ODC activity among the placental and endometrial tissues examined between Days 30 and 140 of gestation ($P < 0.01$). Placentomal ODC activity increased ($P < 0.01$) approximately 2-fold between Days 30 and 40 of gestation, and then decreased ($P < 0.01$) progressively between Days 40 and 100 of gestation.

Placentomal ODC activity did not differ (P > 0.05) between Days 100 and 140 of gestation.

Arginase activity in placental and endometrial tissues

Relatively high arginase activity was present in ovine placentomes, intercotyledonary placenta and intercaruncular endometrium (Table 4.2). Placentomal arginase activity was the highest (P < 0.01) among the tissues examined between Days 30 and 140 of gestation. In intercotyledonary placenta, arginase activity increased (P < 0.01) between Days 30 and 80 of gestation and declined (P < 0.01) thereafter. In intercaruncular endometrium, arginase activity increased (P < 0.01) between Days 30 and 60 of gestation and then declined (P < 0.01).   In placentomes, arginase activity peaked (P < 0.01) on Day 40 of gestation, and declined (P < 0.01) thereafter. In each of these tissues, arginase activity was the lowest (P < 0.01) on Days 120 to 140 of gestation.

TABLE 4.1. Ornithine decarboxylase activity (nmol/g tissue/h) in ovine placenta and endometrium

	Day of gestation							
	30	40	60	80	100	120	140	SEM
Intercotyledonary placenta	0.58^a	0.57^a	0.35^b	0.32^b	0.34^b	0.32^b	0.35^b	0.04
Placentome	1.79^b	3.54^a	1.50^{bc}	1.44^c	0.56^d	0.49^d	0.47^d	0.13
Intercaruncular endometrium	0.60^b	0.82^a	0.56^b	0.54^b	0.34^c	0.32^c	0.35^c	51

[a-d] Data are the mean with pooled SEM values for four ewes per gestational age. Means sharing different superscript letters within a row are different ($P < 0.01$). Enzyme activity was measured in the presence of 0.2 mM ornithine.

TABLE 4.2. Arginase activity (nmol/g tissue/h) in ovine placenta and endometrium

	Day of gestation							
	30	40	60	80	100	120	140	SEM
Intercotyledonary placenta	172[b]	225[a]	232[a]	238[a]	166[b]	122[c]	114[c]	7
Placentome	425[e]	953[a]	760[b]	687[c]	515[d]	441[e]	420[e]	17
Intercaruncular endometrium	257[b]	441[a]	435[a]	246[b]	239[b]	177[c]	158[c]	7

[a-e] Data are the mean with pooled SEM values for four ewes per gestational age. Means sharing different superscript letters within a row are different ($P < 0.01$). Enzyme activity was measured in the presence of 10 mM arginine.

Arginase activity in allantoic and amniotic fluids

As summarized in Table 4.3, marked changes in arginase activity occurred in allantoic and amniotic fluids during gestation. In allantoic fluid, arginase activity in presence of 1mM of arginine was similar between Days 30 and 80, declined on Day 100, and increased again on Day 120, and decreased progressively with the lowest values on Day 140 of gestation, whereas arginase activity in presence of 10mM of arginine peaked on Day 30, the lowest value on Day 80, and increased again between Days 80 and 120 of gestation. Total arginase activities in the presence of 1mM and 10mM of arginine peaked on Day 120 of gestation in allantoic fluid. In amniotic fluid, arginase activity in presence of 1mM of arginine was the lowest values on Days 40 and 60, increased on Day 80, decreased on Days 100, and increased again on Day 120 and 140 of gestation, whereas arginase activity in presence of 10mM of arginine increased between Days 30 and 80, declined on Day 100, and increased again between Days 100 and 140 of gestation. Total arginase activities in presence of 1mM and 10mM of arginine were the highest values on Days 80 and 140 of gestation in amniotic fluid.

TABLE 4.3. Arginase activity in allantoic and amniotic fluids

Day	Allantoic Fluid				Amniotic fluid			
	nmol/ml/min		nmol/min		nmol/ml/min		nmol/min	
	1 mM	10 mM	1 mM	10 mM	1 mM	10 mM	1 mM	10 mM
30	0.14	1.31	3.69	40	0.21	0.33	0.42	0.73
40	0.12	0.43	1.58	4.09	0.08	0.46	1.78	11
60	0.11	0.50	3.09	15.49	0.09	0.43	18	80
80	0.13	0.20	5.92	9.60	0.50	1.91	284	1055
100	0.07	0.42	14	89.16	0.18	1.06	103	586
120	0.19	0.89	93	441	0.49	1.44	60	299
140	0.04	0.39	17	158	0.68	3.05	261	1219
SEM	0.03	0.12	9.87	50	0.08	0.40	24	133

Data are means $\pm$ SEM for 4 ewes per gestational age. For each gestational age, total activity of arginase in amniotic and allantoic fluids was calculated by multiplying arginase activity by amniotic and allantoic fluid volumes, respectively.

Polyamine synthesis in placental and endometrial tissues

Data regarding polyamine synthesis from [^{14}C]ornithine and [^{14}C]arginine are summarized in Tables 4.4 and 4.5, respectively. Rates of polyamine synthesis from [^{14}C]ornithine or [^{14}C]arginine were greatest in placentomes compared with other sites on all Days of gestation (P < 0.01). Polyamine synthesis from ornithine peaked (P < 0.01) on Day 40 of gestation in placentomes, intercaruncular endometrium, and intercotyledonary placenta. In each of these tissues, polyamine synthesis from arginine peaked (P < 0.01) on Day 40 of gestation and declined (P < 0.01) thereafter, with the lowest values on Days 120 to 140. Rates of putrescine, spermidine and spermine synthesized from ornithine or arginine varied greatly with placental and endometrial tissues as well as gestational ages. In placentomes, spermidine and spermine were the major polyamines synthesized from ornithine between Days 30 and 40 and

TABLE 4.4. Polyamine synthesis (pmol/g tissue/3 h) from ornithine in ovine placenta and endometrium

| | Day of gestation | | | | | | | |
	30	40	60	80	100	120	140	SEM
	Intercotyledonary placenta							
Putrescine	170^a	190^a	121^b	127^b	82^c	87^c	75^c	5
Spermidine	223^a	206^a	135^b	113^b	129^b	120^b	124^b	7
Spermine	130^b	204^a	103^c	106^c	126^b	142^b	128^b	6
Total	523^a	598^a	359^b	345^b	337^b	349^b	327^b	16
	Placentome							
Putrescine	363^{bc}	694^a	424^b	343^c	133^d	117^d	124^d	15
Spermidine	624^b	1044^a	508^c	441^d	248^e	255^e	221^e	12
Spermine	648^b	1171^a	487^c	449^c	227^d	214^d	202^d	29
Total	1635^b	2909^a	1419^{bc}	1233^c	608^d	586^d	546^d	56
	Intercaruncular endometrium							
Putrescine	150^b	202^a	146^b	135^b	87^c	89^c	77^c	7
Spermidine	226^b	369^a	218^{bc}	197^c	152^d	101^d	113^d	8
Spermine	221^b	281^a	241^b	236^b	146^c	152^c	162^c	8
Total	598^b	853^a	603^b	567^b	385^c	341^c	352^c	20

[a-e] Data are the mean with pooled SEM values for four ewes per gestational age. Means sharing different superscript letters within a row are different ($P < 0.01$).

TABLE 4.5. Polyamine synthesis (pmol/g tissue/3 h) from arginine in ovine placenta and endometrium

	Day of gestation							
	30	40	60	80	100	120	140	SEM
Intercotyledonary placenta								
Putrescine	21^b	27^a	20^{bc}	18^{bc}	17^c	12^d	12^d	1
Spermidine	26^b	36^a	23^b	25^b	20^c	16^d	15^d	1
Spermine	15^c	32^a	24^b	22^b	16^c	14^c	13^c	1
Total	62^b	95^a	67^b	65^c	53^d	42^e	40^e	3
Placentome								
Putrescine	113^b	224^a	108^b	107^b	56^c	42^d	38^d	3
Spermidine	132^c	326^a	176^b	129^c	89^d	63^e	60^e	6
Spermine	120^c	365^a	192^b	171^b	93^d	66^e	68^e	6
Total	365^c	915^a	477^b	407^c	237^d	182^e	166^e	11
Intercaruncular endometrium								
Putrescine	23^c	41^a	32^b	21^c	15^d	14^d	13^d	1
Spermidine	31^c	69^a	44^b	26^d	21^e	17^f	16^f	1
Spermine	25^e	57^a	45^b	39^c	30^d	24^e	23^e	1
Total	78^c	167^a	121^b	86^c	65^d	54^e	52^e	2

[a-e] Data are the mean with pooled SEM values for four ewes per gestational age. Means sharing different superscript letters within a row are different ($P < 0.01$).

between Days 80 and 140 of gestation, whereas rates of putrescine, spermidine, and spermine synthesis were similar on Day 60 of gestation. In intercaruncular endometrium, spermidine and spermine were the major polyamines synthesized from ornithine and arginine between Days 30 and 100 of gestation, whereas rates of spermine synthesis were highest on Days 120 to 140. In intercotyledonary placenta, rates of putrescine, spermidine and spermine synthesized were similar between Days 40 and 80 of gestation, whereas spermidine and spermine were the major polyamines synthesized between Days 100 and 140 of gestation.

Polyamine concentrations in placental and endometrial tissues

Data are summarized in Table 4.6. There were biphasic changes in total polyamine concentrations in intercotyledonary placenta during pregnancy, as values were similar ($P > 0.05$) between Days 30 and 40 of gestation, increased ($P < 0.01$) approximately 2-fold on Days 60 to 80, declined ($P < 0.01$) on Day 100, and increased again ($P < 0.01$) on Days 120 to 140 to values similar to those on Days 60 to 80 of gestation. Concentrations of total polyamines in intercaruncular endometrium were 41-45% higher ($P < 0.01$) on Days 40 to 60 compared with Day 30 of gestation, and they progressively declined ($P < 0.01$) between Days 60 and 100 of gestation. Placentomes exhibited the highest ($P < 0.01$) concentrations of polyamines among the ovine placental and endometrial tissues examined between Days 30 and 120 of gestation. Placentomal concentrations of total polyamines increased ($P < 0.01$) by 113% between Days 30 and 40 of gestation and decreased ($P < 0.01$) markedly between Days 40 and 80 of gestation. There were no changes ($P > 0.05$) in placentomal concentrations of total polyamines between Days 80 and 120 of gestation. Concentrations of polyamines were similar ($P > 0.05$) among intercotyledonary placenta, intercaruncular endometrium, and placentomes on Day 140 of gestation.

TABLE 4.6. Concentration (nmol/g tissue) of polyamines in ovine placenta and endometrium

	Day of gestation							
	30	40	60	80	100	120	140	SEM
Intercotyledonary placenta								
Putrescine	91[b]	85[b]	154[a]	170[a]	81[b]	93[b]	95[b]	8
Spermidine	90[c]	93[c]	192[a]	182[a]	92[c]	145[b]	185[a]	9
Spermine	73[c]	82[c]	135[b]	171[a]	84[c]	170[a]	196[a]	8
Total	254[c]	260[c]	481[a]	523[a]	257[c]	408[b]	476[a]	23
Placentome								
Putrescine	314[b]	665[a]	387[b]	152[c]	169[c]	144[c]	115[d]	25
Spermidine	477[c]	1039[a]	682[b]	274[d]	349[d]	288[d]	231[d]	32
Spermine	506[c]	1064[a]	710[b]	353[d]	387[d]	367[d]	191[e]	51
Total	1298[c]	2767[a]	1779[b]	778[d]	904[d]	799[d]	537[e]	96
Intercaruncular endometrium								
Putrescine	226[c]	298[b]	356[a]	180[d]	116[e]	124[e]	127[e]	7
Spermidine	355[c]	554[a]	447[b]	314[c]	244[d]	229[d]	244[d]	16
Spermine	329[c]	505[a]	512[a]	442[b]	260[d]	258[d]	239[d]	20
Total	910[b]	1357[a]	1316[a]	936[b]	620[c]	611[c]	594[c]	47

[a-d] Data are the mean with pooled SEM values for four ewes per gestational age. Means sharing different superscript letters within a row are different ($P < 0.01$).

TABLE 4.7. Concentrations (nmol/g tissue) of ornithine and arginine in ovine placenta and endometrium

| | Day of gestation | | | | | | | |
	30	40	60	80	100	120	140	SEM
Intercotyledonary placenta								
Ornithine	316^c	537^b	852^a	906^a	328^c	322^c	340^c	34
Arginine	1003^e	1217^d	1618^b	1773^b	1994^a	1407^c	1025^e	72
Placentome								
Ornithine	183^e	1719^a	964^b	613^c	336^d	324^d	378^d	78
Arginine	564^e	3027^a	1797^c	1848^c	2357^b	1325^d	1244^d	104
Intercaruncular endometrium								
Ornithine	422^c	897^a	577^b	395^c	296^d	288^d	301^d	36
Arginine	813^c	1055^b	807^c	886^{bc}	1569^a	1051^b	799^c	59

[a-d] Data are the mean with pooled SEM values for four ewes per gestational age. Means sharing different superscript letters within a row are different ($P < 0.01$).

Ornithine and arginine concentrations in placental and endometrial tissues

Concentrations of arginine were higher (P < 0.01) than those of ornithine in all placental and endometrial tissues examined between Days 30 and 140 of gestation (Table 4.7). Placentomal arginine and ornithine concentrations increased (P < 0.01) by 437% and 840% between Days 30 and 40 of gestation, respectively, and declined (P < 0.01) thereafter. In intercotyledonary placenta, concentrations of ornithine increased (P < 0.01) progressively between Days 30 and 60-80 of gestation and declined (P < 0.01) thereafter, whereas concentrations of arginine increased progressively between Days 30 and 100 of gestation and then decreased (P < 0.01) progressively between Days 100 and 140 of gestation. In intercaruncular endometrium, concentrations of ornithine increased (P < 0.01) between Days 30 and 40 of gestation and declined (P < 0.01) thereafter, whereas concentrations of arginine increased (P < 0.01)

between Days 30 and 100 of gestation and decreased ($P < 0.01$) progressively during late gestation. Interestingly, arginine concentrations peaked ($P < 0.01$) on Day 100 of gestation in both intercotyledonary placenta and intercaruncular endometrium. Also, placentomal arginine concentrations were higher ($P < 0.01$) on Day 100 of gestation, compared with earlier (Days 30 and 60-80) and later (Days 120-140) stages of gestation.

Polyamine concentrations in allantoic and amniotic fluids

Data are summarized in Table 4.8. Concentrations of total polyamines in ovine allantoic fluid were higher than ($P < 0.01$), similar to ($P > 0.05$), and lower than ($P < 0.01$), those in amniotic fluid on Days 30 to 80, 100, and 120 to 140, respectively. Concentrations of total polyamines in allantoic fluid increased ($P < 0.01$) progressively between Days 30 and 60 of gestation, and declined ($P < 0.01$) during the remainder of pregnancy, with the lowest values being observed on Days 120 to 140 of gestation. Concentrations of total polyamines in amniotic fluid increased ($P < 0.01$) progressively between Days 30 and 100 of gestation, and then declined ($P < 0.01$) during late gestation (Days 120 to 140). In allantoic or amniotic fluids, concentrations of polyamines did not differ ($P > 0.05$) between Days 120 and 140 of gestation. Concentrations of putrescine, spermidine and spermine in allantoic and amniotic fluids varied greatly with day of gestation. For example, concentrations of putrescine in allantoic fluid were greater than ($P < 0.01$), similar to ($P > 0.05$), and lower than ($P < 0.01$), those of spermidine and spermine on Days 30 to 40, 60 to 80, and 100 to 140 of gestation, respectively. In addition, concentrations of putrescine in amniotic fluid were greater than ($P < 0.01$) those of spermine, similar to ($P > 0.05$) and lower than ($P < 0.01$) those of spermidine and spermine on Days 30, 40-60, and 100 of gestation, respectively.

TABLE 4.8. Concentrations (nmol/L) of polyamines in ovine allantoic and amniotic fluids

		Day of gestation							
		30	40	60	80	100	120	140	SEM
Allantoic fluid	Putrescine	2088[b]	3090[a]	3468[a]	1348[c]	545[d]	433[d]	423[d]	203
	Spermidine	1055[d]	1898[b]	3063[a]	1445[c]	2748[a]	795[e]	618[e]	167
	Spermine	923[d]	2155[b]	3260[a]	1643[c]	1195[d]	758[e]	865[de]	106
	Total	4065[c]	7143[b]	9790[a]	4435[c]	4488[c]	1985[d]	1905[d]	285
Amniotic fluid	Putrescine	480[e]	813[bc]	995[a]	773[cd]	698[d]	905[ab]	278[f]	50
	Spermidine	853[bc]	1060[b]	823[bc]	1745[a]	1978[a]	740[c]	545[d]	103
	Spermine	335[e]	1000[d]	1153[d]	1035[d]	1800[c]	2297[b]	2993[a]	132
	Total	1668[d]	2873[c]	2970[c]	3553[b]	4475[a]	3942[b]	3815[al]	161

[a-f] Data are the mean with pooled SEM values for four ewes per gestational age. Means sharing different superscript letters within a row are different ($P < 0.01$).

Data regarding allantoic and amniotic fluid volumes as well as fetal weights of the same sheep as used for the present study have been published previously (Kwon et al., 2003a). The volumes of ovine allantoic fluid averaged 31, 11, 31, 46, 199, 491, and 366 ml, respectively, on Days 30, 40, 60, 80, 100, 120, and 140 of gestation. On the same days, the volumes of ovine amniotic fluid averaged 2.2, 23.8, 190, 543, 595, 383, and 449 ml, respectively. These data, along with concentrations of polyamines in allantoic and amniotic fluids (Table 4.8), were used to calculate the total content of polyamines in these two fetal fluid compartments (Table 4.9). Total content of polyamines in allantoic fluid was greater than ($P < 0.01$), similar to ($P > 0.05$), and lower than ($P < 0.01$), that in amniotic fluid on Days 30, 40, and 60 to 140 of gestation, respectively. Total content of polyamines in allantoic and amniotic fluids peaked ($P < 0.01$) on Day

TABLE 4.9. Total content (nmol) of polyamines in ovine allantoic and amniotic fluids

		Day of gestation							
		30	40	60	80	100	120	140	SEM
Allantoic fluid	Putrescine	64[d]	34[e]	108[c]	62[d]	109[c]	214[a]	155[b]	11
	Spermidine	31[f]	23[f]	96[d]	67[e]	548[a]	393[b]	227[c]	18
	Spermine	30[e]	26[e]	102[d]	76[d]	240[c]	371[a]	316[b]	15
	Total	125[e]	83[f]	306[c]	205[d]	895[a]	978[a]	670[b]	66
Amniotic fluid	Putrescine	1[f]	19[e]	189[c]	420[a]	416[a]	345[b]	126[d]	24
	Spermidine	2[g]	25[f]	157[e]	949[b]	1178[a]	283[c]	245[d]	76
	Spermine	1[f]	24[e]	218[d]	563[c]	1072[b]	882[b]	1344[a]	129
	Total	4[f]	68[e]	564[d]	1931[b]	2667[a]	1510[c]	1715[bc]	195

[a-g] Data are the mean with pooled SEM values for four ewes per gestational age. Means sharing different superscript letters within a row are different ($P < 0.01$).

100 of gestation. Total content of spermine in amniotic fluid increased ($P < 0.01$) progressively with advancing gestation. On the basis of ovine fetal weights, which averaged 0.88, 5.0, 61, 312, 1005, 2322, 4125 g on Days 30, 40, 60, 80, 100, 120, and 140 of gestation, respectively (Kwon et al., 2003a), total content of spermine in amniotic fluid was positively correlated with ovine fetal weights between Days 30 and 140 of gestation ($r = 0.88$, $P < 0.01$).

Placental and endometrial ODC and arginase activities measured with the same substrate concentration (2 mM)

Intercotyledonary placenta, placentomes, and intercaruncular endometrium obtained on Days 40 and 140 of gestation were analyzed for ODC

and arginase activities in the presence of the same substrate concentration (2 mM) (Table 4.10). Placental and endometrial ODC activities measured in the presence of 2 mM ornithine were about 1.4 times greater than those obtained in the presence of 0.2 mM ornithine (Table 4.1), whereas arginase activities measured in the presence of 2 mM arginine were approximately 30% of the values obtained in the presence of 10 mM arginine (Table 4.2). At the same substrate concentration, arginase activity was 60- to 180-fold higher than ODC activity in ovine placental and endometrial tissues (P < 0.01).

TABLE 4.10.   Ornithine decarboxylase (ODC) and arginase activities (nmol/g tissue/h) in ovine placenta and endometrium

	Day 40 of gestation		Day 140 of gestation	
	ODC	Arginase	ODC	Arginase
Intercotyledonary placenta	0.82 ± 0.06	64 ± 3 *	0.48 ± 0.03	33 ± 2 *
Placentome	4.97 ± 0.35	304 ± 12 *	0.67 ± 0.04	120 ± 6 *
Intercaruncular endometrium	1.16 ± 0.09	136 ± 7 *	0.49 ± 0.03	51 ± 3 *

Data are means ± SEM, n = 4.   * P < 0.01 vs ODC activity. ODC and arginase activities were measured in the presence of 2 mM ornithine and 2 mM arginine, respectively.

Correlations between ODC activity and polyamine synthesis or polyamine concentrations in placental and endometrial tissues

These data are summarized in Table 4.11. In placentomes and intercaruncular endometrium, ODC activities were positively correlated (P < 0.01) with both rates of polyamine synthesis and concentrations of polyamines. In both tissues, concentrations of polyamines were positively correlated with rates of polyamine synthesis. Likewise, in intercotyledonary placenta, ODC activities were positively correlated (P < 0.01) with rates of putrescine,

spermidine and total polyamine synthesis. Interestingly, ODC activities were negatively correlated (P < 0.05) with concentrations of spermine, while rates of spermidine, spermine and total polyamine synthesis were also negatively correlated (P < 0.05) with their concentrations in intercotyledonary placenta.

TABLE 4.11. Correlations between ornithine decarboxylase activity (ODC) and polyamine synthesis (PAS) or polyamine concentrations ([PA]) in ovine placenta and endometrium

		ODC vs PAS	ODC vs [PA]	[PA] vs PAS
Intercotyledonary placenta	Putrescine	0.480 *	0.204 NS	0.005 NS
	Spermidine	0.527 *	0.325 NS	-0.608 *
	Spermine	0.335 NS	-0.406 †	-0.390 †
	Total	0.523 *	0.357 NS	-0.599 *
Placentome	Putrescine	0.937 *	0.904 *	0.903 *
	Spermidine	0.947 *	0.823 *	0.859 *
	Spermine	0.940 *	0.769 *	0.819 *
	Total	0.953 *	0.834 *	0.882 *
Intercaruncular endometrium	Putrescine	0.669 *	0.556 *	0.754 *
	Spermidine	0.697 *	0.718 *	0.890 *
	Spermine	0.696 *	0.423 †	0.809 *
	Total	0.708 *	0.594 *	0.854 *

Values are Pearson correlation coefficients, n = 28. † P < 0.05; * P < 0.01. NS, not significant.

Discussion

To our knowledge, this is the first report of changes in polyamine synthesis and concentrations in ovine placental and endometrial tissues during pregnancy. The present study has four major findings: 1) maximal activities of ODC and arginase as well as the highest concentrations of polyamines were expressed in ovine placentomes, intercotyledonary placenta, and intercaruncular endometrium in the first half of pregnancy; 2) the highest concentrations of polyamines in ovine allantoic and amniotic fluids occurred in early and late gestation, respectively; 3) concentrations of putrescine, spermidine and spermine varied greatly among ovine placental and endometrial tissues, fetal fluids, and day of gestation; and 4) arginine was an important precursor for polyamine synthesis in the ovine placenta and endometrium.

Despite the previous reports of changes in rat placental ODC activity during pregnancy (Guha and Janne, 1976; Hoshiai et al., 1981), little is known about polyamine synthesis or concentrations of its physiological substrates in placenta or endometrium of any species. Results of the present study demonstrate clearly that ODC activity, polyamine synthesis from ornithine or arginine, and polyamine concentrations were maximal in ovine placentomes on Day 40 of gestation (Tables 4.1-4.2 and 4.4-4.6) when placental growth and placentomal development are most rapid (Alexander, 1964; Reynolds and Redmer, 1995). ODC activity and polyamine synthesis in intercotyledonary placenta and intercaruncular endometrium, as well as endometrial concentrations of polyamines, also peaked on Day 40 of gestation, when these tissues undergo marked morphological and functional changes (Wimsatt 1950; Stewart et al., 2000; Gray et al., 2001). On the basis of water content of the ovine placentome (approximately 81%), intercotyledonary placenta (approximately 87%) and intercaruncular endometrium (approximately 74%) (our unpublished data), concentrations of polyamines in these tissues were estimated to be 0.66-3.5 mM, 0.29-0.61 mM, and 0.82-1.8 mM, respectively. These values are substantially higher than concentrations of polyamines in maternal or fetal

plasma (2-5 μM) (our unpublished data), indicating the abundance of polyamines in ovine placental and endometrial tissues. Our results suggest an important role for polyamines in placental and endometrial growth and therefore fetal growth. In support of this view, an inhibition of ODC activity during pregnancy markedly reduced placental size and birth weights of rats (Ishida et al., 2002).

A novel finding of this study is the relatively high arginase activity in ovine placental and endometrial tissues (Table 4.2). In addition to ODC, the arginine-derived ornithine can be catabolized by ornithine aminotransferase to yield pyrroline-5-carboxylate (P5C), a substrate for the synthesis of glutamate and proline by mitochondrial P5C dehydrogenase and cytosolic P5C reductase, respectively (Wu and Morris, 1998). Thus, through the formation of ornithine, arginase would be expected to play a regulatory role in polyamine synthesis from arginine in the ovine placenta and endometrium, as recently reported for endothelial cells, vascular smooth muscle cells, and activated macrophages (Wu and Morris, 1998; Li et al., 2001; Wei et al., 2001). Indeed, our results demonstrate that arginine was actively converted into polyamines in ovine placental and endometrial tissues (Table 4.5) and those rates of polyamine synthesis were closely correlated with arginase activity in these tissues (Table 4.2). Ovine allantoic fluid is rich in ornithine and arginine (Kwon et al., 2003a), and is likely a major source of these two amino acids for placenta and endometrium. This view is supported by our findings that the highest concentrations of arginine in ovine allantoic fluid on Day 100 of gestation (Kwon et al., 2003a) were associated with the highest concentrations of arginine in intercotyledonary placenta and intercaruncular endometrium (Table 4.7) and with elevated concentrations of arginine in placentomes (Table 4.7), because probably arginase activity in allantoic fluid was low value on Day 100 of gestation (Table 4.3). On the basis of water content of ovine placentomes, intercotyledonary placenta and intercaruncular endometrium, concentrations of ornithine in these tissues were estimated to be 0.23-2.1 mM, 0.36-1.0 mM, and

0.39-1.2 mM, respectively, and concentrations of arginine in these tissues were estimated to be 0.70-3.7 mM, 1.2-2.3 mM, and 1.1-2.1 mM, respectively. These values are substantially higher than concentrations of ornithine and arginine in ovine maternal plasma (0.04-0.2 mM) and fetal plasma (0.1-0.6 mM) (Kwon et al., 2003a). Interestingly, between Days 30 and 80 of gestation, increases in substrate concentrations and polyamine synthesis in ovine placental and endometrial tissues (Tables 4.4-4.6) are associated with increases in ornithine and arginine concentrations in ovine allantoic fluid (Kwon et al., 2003a) because arginase activity in allantoic fluid was higher between Days 30 and 80 of gestation than on Days 100 and 140 of gestation (Table 4.3), further supporting the earlier suggestion that this fetal fluid is an important nutrient reservoir during gestation (Bazer, 1989). Collectively, our results indicate that arginine is a major source of the ornithine used for polyamine synthesis in ovine placental and endometrial tissues.

The ODC and arginase assay conditions, which involved 0.2 mM ornithine (about 2 times the Km value of the mammalian ODC for ornithine) (Tabor and Tabor, 1984) and 10 mM arginine (about 2 times the Km value of the mammalian arginase for arginine) (Wu and Morris, 1998), respectively, were commonly found in the literature (e.g., Guha and Janne, 1976; Hoshiai et al., 1981; Wu et al., 1998b; Wei et al., 2001). The higher arginase activity (Table 4.2) compared to ODC activity (Table 4.1) in ovine placental and endometrial tissues was not caused by the inclusion of higher substrate (arginine) concentrations in assay solutions. For example, when ODC and arginase activities were determined using the same substrate concentrations (2 mM), which were similar to ornithine and arginine concentrations in the placentomes on Day 40 of gestation (Table 4.7), we found that arginase activity was also much higher than ODC activity in ovine placental and endometrial tissues (Table 4.10). Importantly, results of this study indicate that changes in ODC and arginase activities in ovine placental and endometrial tissues during pregnancy

are consistent with changes in rates of polyamine synthesis from ornithine and arginine, respectively.

Tissue concentrations of polyamines depend on the balance between rates of their synthesis and catabolism (Wu and Morris, 1998). Polyamine synthesis is regulated by ODC and arginase activities as well as the availability of ornithine and arginine (Wu and Morris, 1998; Li et al., 2001; Wei et al., 2001). Thus, ODC activity was positively correlated with polyamine concentrations in placentomes and endometrium (Table 4.11). In addition, concentrations of both ornithine and polyamines peaked between Days 60 and 80 of gestation in intercotyledonary placenta (Tables 4.5 and 4.6). These data further support an important role for polyamine synthesis in regulating polyamine concentrations. In mammalian cells, ODC degradation is catalyzed by the 26S proteasome via an ubiquitin-independent pathway and is greatly accelerated by its association with the polyamine-induced regulatory protein antizyme (Hoyt et al., 2003). Spermidine/spermine N^1-acetyltransferase converts spermine and spermidine to acetyl-spermine and N^1-acetyl-spermidine, respectively. Acetyl-spermine and N^1-acetyl-spermidine are catalysed by polyamine oxidase to form spermidine and putrescine, respectively. The latter is oxidized by diamine oxidase to form succinate plus ammonia. At present, little is known about developmental changes of the ODC antizyme or polyamine-degrading enzymes in ovine tissues. Future studies are necessary to obtain such information to fully explain the developmental changes in polyamine concentrations in ovine tissues (including intercotyledonary placenta) during pregnancy.

Amniotic and allantoic fluid derive, in part, from secretions and transport of water across the placenta and endometrium. Thus, increases in polyamine concentrations in allantoic fluid between Days 30 and 60 of gestation (Table 4.8) were closely correlated with increases in polyamine synthesis in ovine placentomes and endometrium (Tables 4.4 and 4.5). With the development of intestinal polyamine transport systems during gestation, the intake of amniotic fluid by the fetus provides a source of polyamines for supporting proliferation

and differentiation of intestinal epithelial cells.   This is consistent with the increasing content of total polyamines in amniotic fluid with advancing gestation (Table 4.9) and increasing with total activities of arginase in amniotic fluid with advancing gestation (Table 4.3).   The nutritional significance of amniotic fluid is graphically illustrated by the finding that esophageal ligation, which prevents the entry of this fluid into the small intestine, results in intrauterine growth retardation in fetal sheep (Trahair and Harding, 1995).   Although early anatomical studies suggested that the allantoic sac served as a reservoir for fetal wastes, it is now clear that allantoic fluid nutrients may be absorbed by the allantoic epithelium into the fetal-placental circulation and utilized by fetal tissues (Bazer, 1989).   The increases in concentrations of polyamines (Table 4.8) and substrates (ornithine and arginine) for polyamine synthesis (Kwon et al., 2003a) in allantoic fluid during early gestation may play an important role in fetal growth and development.

Our findings indicate that patterns of polyamine synthesis and substrate availability vary greatly with ovine placental and endometrial tissues. Whatever the differences, the highest concentrations of polyamines in these tissues (Tables 4.1-4.2 and 4.4-4.6) occur when their growth is the most rapid during gestation (Alexander, 1964; Bazer, 1979; Atkinson et al., 1984; Reynolds and Redmer, 1995; Taylor et al., 2000).   Importantly, our results reveal metabolic coordination among the several integrated pathways that support high rates of polyamine synthesis in placental and endometrial tissues.   For example, placentomal arginase activity and arginine concentrations peaked on Day 40 of gestation (Table 4.2), thus maximizing the hydrolysis of arginine to ornithine and markedly increasing intracellular concentrations of ornithine (Table 4.7).   The latter was coupled with the highest ODC activity for polyamine synthesis (Table 4.1), thereby maximizing polyamine concentrations (Table 4.6) in ovine placentomes during early gestation.   Similarly, arginase and ODC activities, intracellular ornithine concentrations, and polyamine synthesis from both ornithine or arginine were maximal in intercaruncular endometrium on Day 40 of

gestation, resulting in highest concentrations of polyamines (Table 4.6). Furthermore, concentrations of glutamine, a major substrate for ornithine and arginine synthesis (Wu and Morris, 1998) and a stimulator of ODC activity (Wu et al., 2000c), were highest in ovine allantoic fluid between Days 40 and 60 of gestation (Kwon et al., 2003a).

The present findings raise important questions regarding the physiologic significance of polyamine synthesis in fetal-placental nutrition and development. In this regard, it is noteworthy that maternal undernutrition decreases ornithine concentrations in porcine fetal plasma and allantoic fluid and impairs fetal growth (Wu et al., 1998a; Osgerby et al., 2002), and may also program permanent structural, metabolic and functional alterations (Barker and Clark, 1997; Symonds et al., 2001). Maternal undernutrition in sheep (50% of NRC nutrient requirements) from Day 28 to Day 78 of gestation decreased concentrations of putrescine, spermidine and spermine in amniotic and allantoic fluids by 38-43% (our unpublished data), and reduced fetal growth by 32% on Day 78 of gestation (Vonnahme et al., 2003). Because recent epidemiological studies in humans suggest that the existence between intrauterine growth retardation and development of chronic disease (e.g., diabetes, hypertension, and coronary heart disease) later in life (Barker and Clark, 1997; Symonds et al., 2001), placental synthesis of polyamines may have important implications for both intrauterine growth retardation and fetal origins of diseases in adults.

As noted previously, physiological concentrations of polyamines (putrescine, spermidine and spermine) are key regulators of angiogenesis, early embryogenesis, placental trophoblast growth, and embryonic development in the uterus (Forzard et al., 1980; Henningsson et al., 1982). Results of the present study indicate that polyamine synthesis and concentrations were the highest in ovine placentomes and endometrium on Day 40 of gestation when their growth and morphological changes were most rapid. Relatively high levels of polyamine concentrations were present in ovine placental and endometrial tissues in the second half of pregnancy when there is continued development of

the placental vascular bed and increases in total uterine blood flow to support fetal growth (Ford, 1995; Reynolds and Redmer, 1995). Importantly, there is metabolic coordination among the several integrated pathways that support high rates of polyamine synthesis in the placenta and endometrium. Our findings provide a new base of information for future studies to define the roles of polyamines in fetal-placental growth and development.

CHAPTER V

DEVELOPMENTAL CHANGES IN NITRIC OXIDE SYNTHESIS

IN THE OVINE PLACENTA

Introduction

The placentae of all mammalian species undergo rapid formation of new blood vessels (angiogenesis) and marked growth during pregnancy (Ford, 1995; Reynolds and Redmer, 2001). 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) (Alexander, 1964; Reynolds and Redmer, 1995). The ovine placenta has 60 to 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 (Alexander, 1964).

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 (Maul et al., 2003). 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 (Alderton et al., 2001). In most tissues, nNOS and eNOS are constitutively

expressed, and are termed constitutive NOS (cNOS), whereas iNOS is induced by inflammatory cytokines and hormones. cNOS, but not iNOS, requires Ca^{2+} for enzymatic activity. Both cNOS and iNOS are expressed in placentae of mammals, including humans, pigs, and sheep (Weiner et al.,1994; Magness et al., 1997; Farina et al., 2001; Galan et al., 2001). In ovine placenta, cNOS is composed primarily of eNOS but little nNOS (Magness et al., 1997). Although there are reports of NOS expression and NO synthesis in ovine placenta during late gestation (Days 110-142) (Zheng et al., 2000), 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 (Kwon et al., 2003a). Interestingly, these temporal changes coincide with the period of most rapid growth of the ovine placenta (Alexander, 1964; Reynolds and Redmer, 1995). 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 (Gray et al., 2001).

Materials and Methods

Chemicals.

Tetrahydrobiopterin (BH4), HEPES, N^G-monomethyl-L-arginine, biopterin, NADPH, GTP, arginine, dithiothreitol (DTT), EGTA, EDTA, aprotinin, chymostatin, phenylmethylsulfonylfluoride, pepstatin, FAD, FMN, and calmodulin were purchased from Sigma Chemicals (St. Louis, MO). L-[U-^{14}C]arginine was obtained 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, 200-400mesh) was purchased from Bio-Rad (Richmond, CA) and was converted to the Na^+ from before use as recommended by the manufacturer.

Experimental animals

Columbia cross-bred 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 were marked changes in physiological parameters in the ovine conceptus during pregnancy (e.g. Kwon et al., 2003a, 2003b) and because coefficients of variations for all measured parameters were relatively small (< 5-8%), we used 4 ewes per day of gestation on the basis of statistical power calculation. 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 twin 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 (Kwon et al., 2003a). 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 8:00 and 9:00 A.M., 24 h after the last feeding (Kwon et al., 2003a). All ewes were administered isofluorane (5%) via an inhalation mask to induce anesthesia which was maintained with

isofluorane (1-5%). A mid-ventral 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 week. 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 (BME) containing 0.4 mM L-arginine, 0.5 mM L-glutamine, 5 mM D-glucose, 100 units/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 BME, and then incubated at 37°C for 6 h in 0.5 ml fresh oxygenated BME. Concentrations of other amino acids in the BME 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 (Li et al., 2000). Briefly, 100 μl medium (diluted 1:2) was incubated for 10 min at room temperature with 10 μl of 316 μM 2,3-diaminonapthalene, followed by addition of 5 μl of 2.8 M NaOH. The derivative was separated on a C_8 column (150 X 4.6 mm I.D.) 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 (Li et al., 2000). 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 (Meininger and Wu, 2002). 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 (1 mM DTT, 1 mM $MgCl_2$, 0.1 mM [U-^{14}C] arginine (5×10^3 dpm/nmol), 0.1 mM citrulline, 10 mM valine (an inhibitor of arginase), 0.1 mM NADPH, 0.1 mM BH4, 0.1 mM FAD, 0.1 mM FMN, 10 μg calmodulin, and 0.1 M HEPES, pH 7.4). 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^oC 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 x 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). iNOS activity = (^{14}C

dpm in the iNOS tube – ^{14}C dpm in the blank tube)/[^{14}C]arginine SA). cNOS activity = total NOS activity – iNOS activity.

Determination of GTP-CH activity

GTP-CH activity in placental and endometrial tissues was determined using HPLC (Meininger and Wu, 2002). 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 x 60 mm), 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 x 25 cm, 5 um) 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, 7.5 mM sodium phosphate (pH 6.35).

Determination of BH4

BH4 was analyzed by HPLC as described by Meininger and Wu (2002). 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 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 (Wu et al., 2001). 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 bathophenanthrolinedisulfonic 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 x 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 (Steel et al., 1997), using the PROC GLM and PROC CORR procedures of Statistical Analysis System (SAS, Cary, NC). Differences between means were determined by the Student-Newman-Keuls multiple comparison test following one-way analysis of variance (Steel et al., 1997). 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 5.1). 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. 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 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),

TABLE 5.1. Nitric oxide synthase activities ($nmol \cdot g\ tissue^{-1} \cdot h^{-1}$) 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	0.1
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}	0.2
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

$^{a-f}$ 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). NOS, nitric oxide synthase.

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 5.2. 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 < 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

TABLE 5.2. 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	0.1
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

[a-d] 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).

Day 80 of gestation. In all placental and endometrial tissues, NO synthesis did not differ (P > 0.05) between Days 120 and140 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 5.3). 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)

TABLE 5.3. 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

[a-d] 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).

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 5.4. 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 5.4). In all of these tissues, NADPH levels increased (P < 0.01) progressively between Days 30 and 60 of gestation, declined (P < 0.01) by

TABLE 5.4. Tetrahydrobiopterin and NADPH concentrations (nmol•g tissue^{-1}) 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

[a-d] 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).

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 NADPH levels in intercotyledonary placenta, placentomes, and intercaruncular endometrium (Table 5.5). 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 5.5. Correlations between NO synthesis and NOS activity, GTP-CH activity, BH4 level, and NADPH level in ovine placenta and endometrium

	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 *

Values are Pearson correlation coefficients, n = 28. * $P < 0.01$. BH4, tetrahydrobiopterin; GTP-CH, GTP cyclohydrolase I; NO, nitric oxide; NOS, nitric oxide synthase.

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 (Sladek et al., 1997; Bird et al., 2003; Maul et al., 2003). The available evidence suggests that uterine synthesis of NO increases during pregnancy and decreases at the onset of parturition (Maul et la., 2003). In addition, recent studies with the ovine model demonstrated that NO is a major mediator of placental-fetal blood flow during pregnancy (Rosenfeld et al., 1996). Despite the report of NOS expression and NO synthesis in ovine placenta during late gestation (Days 110-142) (Zheng et al., 2000), 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 (Barker and Clark, 1997) that are related to disturbed NO metabolism (Ozaki et al., 2000; Edwards and McMilen, 2001). Because changes in NOS activity may not necessarily indicate changes in NO production by intact cells (Wu and Morris, 1998; Alderton et al., 2001), 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 (Alderton et al., 2001). 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 tighly 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 (Alderton et al., 2001). Recent evidence shows that BH4 primarily plays a redox role in NOS catalysis, in which BH4 donotes 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 (Wei et al., 2003). 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^{ω}-hydroxyl-L-arginine as an enzyme-bound intermediate. L-Citrulline can be recyled into L-arginine via argininosuccinate synthase and argininosuccinate lyase in virtually all animal cells (Wu and Morris, 1998). 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 $\pm$ 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 $\pm$ SEM, n = 4), at Day 60 of gestation (Wu G, unpublished data). The increase in enzyme activities is associated with increased arginine concentration in ovine allantoic fluid between Days 40 and 60 of gestation (Kwon et al., 2003a).

Because of the intracellular compartmentalization of NO synthesis and its functional consequence (Wu and Morris, 1998), much attention has been directed towards determining NOS isoforms in the female reproductive organs (e.g., Weiner et al., 1994; Magness et al., 1997; Farina et al., 2001; Galan et al., 2001; Maul et al., 2003). Both cNOS and iNOS are present in the gravid rat and ovine uterine tissues and may vary with species and gestational age (Natuzzi et al., 1993; Figueroa and Massmann, 1995; Buhimschi et al., 1996; Ali et al., 1997; Massmann et al., 1999). For example, nNOS and eNOS were more predominant than iNOS in the ovine uterus during the last third of gestation (Massmann et al., 1999), whereas the opposite was reported for the rat uterus throughout pregnancy (Ali et al., 1997). In contrast, cNOS and iNOS activities were similar in both porcine placenta and endometrium during early and mid gestation (Wu et al., 1998b). 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 intercotylednonary 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 (Weiner et al., 1994; Bird et al., 2003), cytokines (Alderton et al., 2001), uterine secretions (Maul et al., 2003), as well as nutrients and their metabolites (Wu and Meininger, 2002).

Placental synthesis of NO, like that of polyamines (other products of arginine catabolism) essential for placental angiogenesis and growth) (Kwon et al., 2003b), increased markedly between Days 30 and 60 of gestation (Table 5.2) when placental growth and placentomal development are most rapid (Alexander, 1964; Reynolds and Redmer, 1995). Likewise, NO synthesis in intercaruncular endometrium peaked on Days 40-60 of gestation, when this tissue undergoes marked morphological and functional changes (Gray et al., 2001). Results of the present study support our hypothesis that NO and polyamines are crucial for placental and endometrial growth during early pregnancy (Kwon et al., 2003b; Maul et al., 2003) and, therefore, for 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 (Diket et al., 1994; Buhimschi et al., 1995; Greenberg et al., 1997; Ishida et al., 2002). 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 (Ford, 1995; Reynolds and Redmer, 1995). 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 (Kwon et al., 2003a). 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 relative constant between Days 40 and 80 of gestation, and then increases exponentially thereafter (Stegeman, 1974; Teasdale, 1976). In contrast, vascular density of caruncular tissues increases substantially until mid-gestation and then more slowly thereafter (Teasdale, 1976).   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 (Rudolph and Heymann, 1970; Reynolds and Ferrell, 1987).   On the basis of these observations, we surmise that the placentomal increase in NO synthesis between Days 30 and 60 and between 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 in order to dtermine 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 (Alderton et al., 2001), including human placenta (Kukor et al., 2000), 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 (Wu et al., 1994), whereas GTP-CH catalyzes the first and rate-controlling step in the *de novo* synthesis of BH4 from GTP (Thöny et al., 2000). 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 5.4), as did allantoic fluid concentrations of citrulline (the precursor of arginine) and arginine (Kwon et al., 2003a). Between Days 80 and 100 of gestation, BH4 concentrations also increased in placentome and endometrium (Table 5.4), as did concentrations of arginine in allantoic fluid (Kwon et al., 2003a). This amino acid, which is a potential regulator of the pentose cycle activity (Wu et al., 1994) and a stimulator of endothelial GTP-CH expression (Wu et al., 2003), 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 5.5).

On the basis of water content of the ovine placentome (approximately 81%), intercotyledonary placenta (approximately 87%) and intercaruncular endometrium (approximately 74%) (Kwon et al., 2003b), 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 Km of purified eNOS and iNOS for NADPH (~ 1 μM) and BH4 (~ 0.1 μM) (Alderton et al., 2001), changes in tissue NADPH and BH4 levels can modulate both constitutive and inducible NO production (Meininger et al., 2000; Wu et al., 2001; Medina et al., 2003; Zheng et al., 2003). 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% (Wu et al., 2001). Furthermore, increasing BH4 levels 140% through GTP-CH gene transfer increased NO production by 260% in carotid arteries of hypertensive rats (Zheng et al., 2003). Similarly, intracellular concentrations of arginine (~1-2 mM) are much higher than the Km of purified eNOS and iNOS for arginine (~ 3 to 20 μM) (Alderton et al., 2001), but increasing tissue arginine above 2 mM stimulates NO production by both endothelial cells and activated macrophages (Wu and Meininger, 2002). This

so-called "paradox of NO synthesis" may be explained by the compartmentalization of BH4, NADPH, and arginine (Wu and Morris, 1998), multiple competitive pathways for their utilization (Medina et al., 2003), and protein-protein interaction in intact cells (Alderton et al., 2001).

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 gestation (Alexander, 1964; Bazer, 1979; Atkinson et al., 1984; Reynolds and Redmer, 1995; Gray et al., 2001). Additionally, our results suggest metabolic coordination among the several integrated pathways that support high rates of NO in placental and endometrial tissues (Figure 5.1). For example, concentrations of citrulline (the effective precursor of arginine) in ovine allantoic fluid increase 34-fold between Days 30 and 60 of gestation (Kwon et al., 2003a), thus increasing the availability of arginine for metabolism in placental and endometrium (Kwon et al., 2003b). In addition, placentomal GTP-CH activity peaked on Day 60 of gestation (Table 5.2), thus maximizing the de novo synthesis of BH4. Consequently, placentomal BH4 and NADPH concentrations were highest on Day 60 of gestation (Table 5.5). 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 (Wu et al., 1998; Osgerby et al., 2002), and may also program permanent structural, metabolic and functional alterations (Barker and Clark, 1997; Symonds et al., 2001). 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
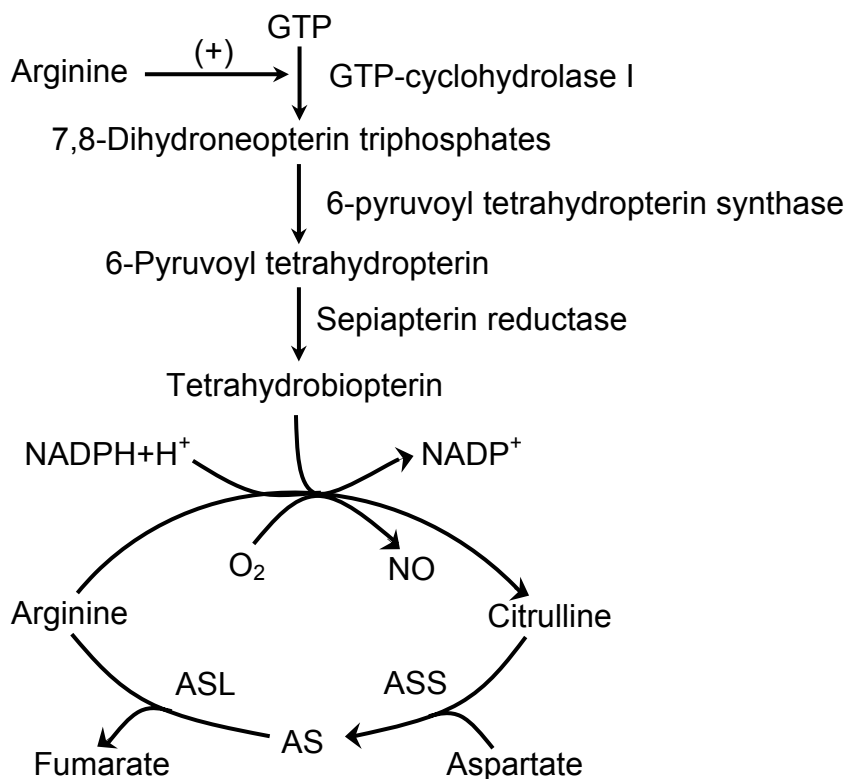
GTP

Arginine $\xrightarrow{\quad(+)\quad}$ GTP-cyclohydrolase I

7,8-Dihydroneopterin triphosphates

$\downarrow$ 6-pyruvoyl tetrahydropterin synthase

6-Pyruvoyl tetrahydropterin

$\downarrow$ Sepiapterin reductase

Tetrahydrobiopterin

NADPH+H$^+$ NADP$^+$

O$_2$ NO

Arginine   Citrulline

ASL   ASS

Fumarate   AS   Aspartate

Fig 5.1.   Role of arginine, tetrahydrobiopterin and NADPH in nitric oxide synthesis.   AS, argininosuccinate; ASL, argininosuccinate lyase; ASS, aegininosuccinate synthase; NO, nitric oxide.   Tetrahydrobiopterin (BH4) primarily plays a redox role in NOS catalysis, in which BH4 donotes 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.

plasma and allantoic fluids by 20-38% (Kwon H, Wu G, Bazer FW, Spencer TE, Ford SP, unpublished data), and reduced fetal growth by 32% on Day 78 of gestation (Vonnahme et al., 2003).   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 (Barker and Clark, 1997; Symonds et al., 2001), 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 polyamines in fetal-placental growth and development.

CHAPTER VI

MATERNAL UNDERNUTRITION REDUCES CONCENTRATIONS OF AMINO ACIDS AND POLYAMINES IN OVINE FETAL PLASMA AND FLUIDS

Introduction

Studies over past decades have established intrauterine growth retardation (IUGR) in response to undernutrition in humans, sheep, pigs, and rats (Widdowson, 1977; Osgerby et al., 2002; Ramachandran, 2002; Vonnahme et al., 2003). Undernutrition in pregnant women may result from low dietary intake of nutrients owing to either a limited supply of foods or severe nausea and vomiting known as hyperemesis gravidarum (Snell et al., 1998). Epidemiological studies in humans suggest that alterations in fetal nutrition and endocrine status may result in developmental adaptations that permanently change the structure, physiology and metabolism of the offspring, thereby predisposing individuals to cardiovascular, metabolic and endocrine diseases in adult life (Barker and Clark, 1997). In domestic animals, undernutrition during gestation also occurs in the U.S. and worldwide. For example, the nutrient intake of grazing ewes in the western United States is often < 50% of the NRC requirement because of significant fluctuations in both quantity and quality of forage due to extreme variations in precipitation (Thomas and Kott, 1995). A recent report indicated that, in India, sheep reared on rangeland are severely underfed for most part of the year because of low biomass (Chaturvedi et al., 2003). Unsupplemented grazing ewes lose a significant amount of body weight from early to mid-gestation and even after supplementation later in gestation, their health, fetal growth and lactation performance are compromised (Thomas and Kott, 1995). Thus, studies of the mechanisms for IUGR brought about by maternal undernutrition have important implications for both human medicine and animal agriculture.

Amino acids play a vital role in development of the conceptus (embryo/fetus and associated placental membranes). In addition to serving as

building blocks for tissue protein synthesis, amino acids function as antioxidants, regulators of hormone secretion, major fuels for fetal growth, and cell signaling molecules (Wu and Morris, 1998; Stipanuk and Watford, 2000; Flynn et al., 2002). Furthermore, amino acids are essential precursors for the synthesis of non-protein substances with enormous versatility and biological importance, including nitric oxide, polyamines, neurotransmitters, amino sugars, purine and pyrimidine nucleotides, creatine, carnitine, porphyrins, melatonin, melanin, and sphingolipids (Stipanuk and Watford, 2000). For example, nitric oxide, a product of arginine catabolism, plays a crucial role in regulating placental angiogenesis and fetal-placental blood flows during gestation (Sladek et al., 1997; Reynolds and Redmer, 2001; Bird et al., 2003). Polyamines (polycationic molecules) regulate gene expression, signal transduction, ion channel function, DNA and protein synthesis, as well as cell proliferation, differentiation, and function (Flynn et al., 2002). Surprisingly, little is known about the effect of maternal undernutrition on concentrations of amino acids and polyamines in the conceptus of any species.

The sheep is a widely used animal model for studying human fetal-placental development (Lemons et al., 1976; Sladek et al., 1997; Wallace, 2000; Bird et al., 2003). We have recently reported striking changes in concentrations of amino acids and polyamines in the ovine conceptus during pregnancy (Kwon et al., 2003a, 2003b). The significance of these changes for normal fetal growth and development may be underscored under a pathophysiological condition (e.g., maternal undernutrition or overnutrition) that is known to cause IUGR. We hypothesized that maternal undernutrition reduces the availability of both amino acids and polyamines in the conceptus. This hypothesis was tested using our established ovine model of maternal undernutrition and IUGR (Vonnahme et al., 2003).

Materials and Methods

Animals

Two series of experiments were conducted with multiparous ewes of mixed breeding (Vonnahme et al., 2003). In both Experiment 1 and 2, maternal undernutrition (50% of NRC nutrient requirements) of ewes between Days 28 and 78 was carried out as described previously (Vonnahme et al., 2003). Briefly, the diet consisted of a pelleted beet pulp (79.7% total digestible nutrient, 93.5% dry matter, 10% crude protein) and a mineral-vitamin mixture. On Day 20 of gestation, 16 ewes were weighed so that individual diets could be calculated on a metabolic body weight basis (weight$^{0.75}$). On Day 21 of gestation, all ewes were placed in individual pens and provided all nutrients that met NRC maintenance requirements for early gestation. On Day 28 of gestation, ewes were assigned randomly to a control group (n = 8) fed 100% of NRC nutrient requirements) or a nutrient-restricted group (n = 8; 50% of NRC nutrient requirements). Every 7 days beginning on Day 28 of gestation, ewes were weighed and rations adjusted for changes in body weight. On Day 45 of gestation, the number of fetuses carried by each ewe was determined by ultrasonography (Ausonics Microimager 1000 sector scanning instrument; Ausonics Pty Ltd, Sydney, Australia). On Day 78 of gestation, each ewe in Experimnet 1 was administered an overdose of sodium pentabarbitol (Abbott Laboratories, Abbott Park, IL) and exsanguinated. After the tip of the gravid uterine horn was exposed, blood samples were withdrawn from uterine artery and umbilical vein into cooled heparinized tubes. The tubes were immediately centrifuged at 4 $^{\circ}$C and 3000 g for 10 min to obtain plasma. All plasma samples were stored at –80 $^{\circ}$C until analyzed. On Day 78 in Experiment 2, nutrient-restricted ewes either continued to be fed 50% of NRC nutrient requirements or realimented to 100% of NRC nutrient requirements. On Day 135 of gestation, blood samples were obtained from uterine artery and umbilical vein of all ewes, as described for Experiment 1.

Fetal weights on Day 78 of gestation were heavier (P<0.05) for the control ewes than for the nutrient-restricted ewes, averaging 326.36±19.99 g and 221.68±7.90 g, respectively. On Day 135 of gestation, fetal weights of control-fed and nutrient-restricted realimented ewes were similar averaging 4782.0±165.1 g and 4639.6±236.4 g, respectively, while fetal weights of continuously undernourished ewes were reduced (P<0.05) when compared to control-fed ewes averaging 4047.0±175.5 g.

Analysis of amino acids

Plasma and fetal fluids were analyzed for amino acids, as described previously (Wu et al., 1997). Briefly, amino acids, except for proline, were determined by fluorometric HPLC methods involving pre-column derivatization with o-phthaldialdehyde. The values for total cysteine in plasma and fluids represent free cysteine plus ½ cystine. Proline was measured using a fluorometric HPLC method involving pre-column derivatization with 9-fluorenylmethyl chloroformate. Amino acids in samples were quantified on the basis of known amounts of standards (Sigma Chemicals, St. Louis, MO) using Millenium-32 Software (Waters, Milford, MA).

Analysis of polyamines

Fetal fluids were analyzed for polyamines by an ion-pairing HPLC method involving precolumn-derivatization with *o*-phthaldialdehyde (Wu et al., 1998b). Putrescine, spermidine and spermine in samples were quantified on the basis of known amounts of standards (Sigma Chemicals), using Millenium-32 Software (Waters, Milford, MA).

Statistics Analysis

Statistical comparisons between the two groups of ewes euthanized on Day 78 of gestation were performed using the unpaired t-test. Statistical comparisons among the three groups of ewes euthanized on Day 135 of

gestation were completed using a one-way analysis of variance. Post hoc analysis was performed with a Tukey's test for highly significant differences. All statistical analyses were performed using the SAS V8.2 program for Windows® (SAS Inst. Inc., Cary, NC), and significance was accepted when $P \le 0.05$.

Results

Maternal undernutrition between Days 28 and 78 of gestation

Table 6.1 summarizes amino acid concentrations in maternal and fetal plasma on Day 78 of gestation. Glycine was the most abundant amino acid in both maternal and fetal plasma. Compared with control-fed ewes, maternal undernutrition between Days 28 and 78 of gestation increased (P < 0.01) plasma concentration of glycine, but decreased (P < 0.05) plasma concentrations of many amino acids (arginine, citrulline, cysteine, glutamine, isoleucine, leucine, ornithine, phenylalanine, proline, serine, and valine) in both maternal and fetal plasma. Nutrient restriction also decreased (P < 0.05) concentrations of lysine, methionine, threonine, tryptophan, and tyrosine in maternal plasma, but had no effect (P > 0.05) on these amino acids in fetal plasma. Underfeeding had no effect (P > 0.05) on concentrations of β-alanine, asparagine, aspartate, glutamate, histidine, and taurine in either maternal or fetal plasma. Concentrations of total α-amino acids were 8.8% and 8.3% lower (P < 0.01), respectively, in maternal and fetal plasma of nutrient-restricted ewes, when compared with control-fed ewes.

Concentrations of amino acids in allantoic and amniotic fluids on Day 78 of gestation are summarized in Table 6.2. Alanine, citrulline, glutamine, glycine, and serine represented 73% and 76% of total α-amino acids in allantoic fluid of control-fed and nutrient-restricted ewes, respectively. Compared with control-

TABLE 6.1. Amino acid concentrations (μM) in ovine maternal and fetal plasma on Day 78 of gestation

Amino acid	Uterine arterial plasma		Fetal umbilical plasma	
	Control	Restricted	Control	Restricted
Ala	229 ± 8	200 ± 7 [†]	405 ± 16	421 ± 17
β-Ala	11 ± 0.6	10 ± 0.5	86 ± 5	83 ± 3
Arg	130 ± 3	100 ± 4 *	223 ± 11	157 ± 5 *
Asn	21 ± 0.9	19 ± 0.8	79 ± 4	80 ± 7
Asp	11 ± 0.8	11 ± 0.7	16 ± 0.9	14 ± 0.7
Cit	175 ± 3	130 ± 7 *	211 ± 9	140 ± 4 *
Cys	168 ± 3	139 ± 5 *	186 ± 4	170 ± 5 [†]
Gln	219 ± 7	156 ± 3 *	437 ± 13	349 ± 10 *
Glu	69 ± 2	65 ± 2	72 ± 4	73 ± 4
Gly	454 ± 17	669 ± 20 *	499 ± 15	772 ± 15 *
His	49 ± 2	46 ± 1.5	55 ± 2	58 ± 3
Ile	65 ± 1.4	46 ± 1.4 *	77 ± 2	64 ± 2 *
Leu	97 ± 4	71 ± 3 *	158 ± 5	128 ± 4 *
Lys	102 ± 3	83 ± 2 *	189 ± 6	175 ± 7
Met	21 ± 0.6	17 ± 0.3 *	63 ± 2	57 ± 2
Orn	53 ± 2	37 ± 1.5 *	114 ± 5	95 ± 3 *
Phe	48 ± 2	37 ± 1.6 *	118 ± 6	100 ± 6 [†]
Pro	111 ± 4	78 ± 3 *	194 ± 4	153 ± 4 *
Ser	75 ± 2	54 ± 2 *	418 ± 16	323 ± 16 *
Taurine	68 ± 3	63 ± 3	131 ± 8	119 ± 7
Thr	71 ± 2	57 ± 2 *	333 ± 9	305 ± 10
Trp	31 ± 2	23 ± 0.7 *	49 ± 1.3	51 ± 1.7
Tyr	52 ± 2	38 ± 1 *	126 ± 8	118 ± 4
Val	164 ± 3	125 ± 4 *	195 ± 5	164 ± 4 *
Total α-AA	2411 ± 21	2200 ± 41 *	4215 ± 49	3865 ± 38 *

Data are means ± SEM. n=8, [†] P < 0.05; * P<0.01. Control, fed 100% of NRC requirements between Days 28 and 78 of gestation. †Fed 50% of NRC nutrient requirements betweens Days 28 and 78 of gestation. AA, amino acids.

TABLE 6.2. Amino acid concentrations (μM) in ovine fetal fluids on Day 78 of gestation

Amino acid	Allantoic fluid		Amniotic fluid	
	Control	Restricted	Control	Restricted
Ala	4183 ± 272	3905 ± 256	43 ± 2	37 ± 2.2
β-Ala	1049 ± 34	1033 ± 65	31 ± 1	28 ± 1.8
Arg	813 ± 24	653 ± 23 *	30 ± 2	23 ± 0.9 *
Asn	374 ± 27	338 ± 23	16 ± 0.8	14 ± 1
Asp	136 ± 9	145 ± 11	6.6 ± 0.4	5.8 ± 0.3
Cit	2541 ± 126	1534 ± 121 *	36 ± 1.7	23 ± 1.1 *
Cys	536 ± 15	469 ± 34	42 ± 2	34 ± 2 †
Gln	4431 ± 264	3218 ± 133 *	76 ± 4	49 ± 2 *
Glu	150 ± 9	151 ± 6	11 ± 0.6	9.5 ± 0.6
Gly	3308 ± 145	7129 ± 408 *	79 ± 3	180 ± 8 *
His	221 ± 20	200 ± 19	4.6 ± 0.3	4.2 ± 0.3
Ile	150 ± 9	119 ± 4 *	5 ± 0.3	3.7 ± 0.2 *
Leu	553 ± 31	453 ± 19 †	39 ± 2	29 ± 1 *
Lys	1076 ± 44	1025 ± 47	54 ± 3	46 ± 2 †
Met	65 ± 3	70 ± 3	4.4 ± 0.2	4 ± 0.2
Orn	386 ± 28	293 ± 12 †	82 ± 6	73 ± 3
Phe	385 ± 21	361 ± 14	27 ± 1.6	24 ± 1.3
Pro	735 ± 23	591 ± 30 *	43 ± 1.8	35 ± 1.3 *
Ser	4296 ± 186	3480 ± 176 *	95 ± 3	65 ± 3 *
Taurine	1383 ± 82	1208 ± 82	92 ± 3	82 ± 3 †
Thr	795 ± 47	765 ± 26	27 ± 1.8	25 ± 1.9
Trp	118 ± 3	111 ± 6	4.8 ± 0.4	4.4 ± 0.2
Tyr	103 ± 5	93 ± 6	5.5 ± 0.4	5 ± 0.3
Val	414 ± 20	349 ± 14 †	25 ± 1.4	20 ± 0.9 †
Total α-AA	25768 ± 596	25449 ± 556	753 ± 11	714 ± 11 †

Data are means ± SEM. n = 8, † P < 0.05 vs control; * P<0.01 vs control. AA, amino acids.

TABLE 6.3. Polaymine concentrations (μM) in ovine fetal fluids on Day 78 of gestation

Polyamine	Allantoic fluid		Amniotic fluid	
	Control	Restricted	Control	Restricted
Putrescine	1469 ± 49	951 ± 33 *	760 ± 20	547 ± 37 *
Spemidine	1544 ± 53	1015 ± 52 *	1753 ± 73	1104 ± 65 *
Spermine	1655 ± 47	994 ± 62 *	1120 ± 72	753 ± 20 *
Total PA	4668 ± 144	2961 ± 121 *	3633 ± 133	2404 ± 61 *

Data are means ± SEM. n = 8, * $P < 0.01$ vs control.

Compared with control-fed ewes, maternal undernutrition between Days 28 and 78 of gestation increased ($P < 0.01$) concentrations of glycine more than 2-fold but decreased ($P < 0.05$) concentrations of many amino acids (arginine, citrulline, glutamine, isoleucine, leucine, proline, serine, and valine) (Table 6.2) as well as polyamines (Table 6.3) in both allantoic and amniotic fluids. Nutrient restriction had no effect ($P > 0.05$) on concentrations of cysteine, lysine, and taurine in allantoic fluid, but decreased ($P < 0.05$) concentrations of these amino acids in amniotic fluid; the opposite was observed for ornithine. Concentrations of alanine, β-alanine, asparagine, aspartate, glutamate, histidine, methionine, phenylalanine, threonine, tryptophan, and tyrosine in allantoic and amniotic fluids did not differ ($P > 0.05$) between control-fed and nutrient-restricted ewes. On Day 78 of gestation, maternal undernutrition had no effect ($P > 0.05$) on concentrations of total α-amino acids in allantoic fluid, but slightly decreased ($P < 0.05$) concentrations of total α-amino acids by 5% in amniotic fluid.

Maternal undernutrition between Days 28 and 135 of gestation

Concentrations of amino acids in maternal and fetal plasma on Day 135

TABLE 6.4. Amino acid concentrations (μM) in ovine maternal and fetal plasma on Day 135 of gestation

Amino acid	Maternal plasma			Fetal plasma		
	Control	Restricted[†]	Realimented[‡]	Control	Restricted[†]	Realimented[‡]
Ala	172± 5[a]	138± 6[b]	194± 14[a]	485± 19[a]	374± 26[b]	448± 16[a]
β-Ala	34± 3	31± 1	29± 2	205± 17	192± 24	198± 12
Arg	184± 8	97± 2[b]	113± 6[b]	309± 18[a]	224± 22[b]	182± 12[b]
Asn	39± 2	26± 2	36± 3	103± 3[a]	69± 5[b]	88± 5[b]
Asp	10± 1	9± 0.3	10± 1	21± 1	18± 1	19± 1
Cit	150± 8[a]	83± 2[b]	79± 5[b]	239± 9[a]	145± 9[b]	150± 7[b]
Cys	149± 4[a]	63± 3[c]	93± 4[b]	186± 8[a]	100± 3[c]	118± 6[b]
Gln	329± 19[a]	244± 6[b]	241± 19[b]	881± 35[a]	753± 25[b]	726± 29[b]
Glu	86± 3	113± 7	89± 6	71± 4[a]	46± 4[c]	63± 3[b]
Gly	565± 22	515± 29	497± 25	1052± 49[a]	635± 40[c]	829± 45[b]
His	47± 2[a]	28± 1[b]	31± 2[b]	73± 4[a]	54± 5[b]	69± 4[a]
Ile	83± 4[a]	67± 2[b]	70± 2[b]	147± 3[a]	106± 3[b]	119± 4[b]
Leu	112± 5[a]	66± 4[c]	80± 3[b]	211± 8[a]	130± 13[b]	161± 8[b]
Lys	139± 6[a]	85± 3[c]	98± 4[b]	200± 9[a]	140± 7[b]	149± 8[b]
Met	27± 1[a]	17± 0.6[c]	21± 1[b]	67± 2[a]	38± 6[c]	52± 2[b]
Orn	41± 3[c]	31± 2[b]	33± 2[b]	143± 8[a]	73± 3[c]	93± 5[b]
Phe	46± 2[a]	27± 2[c]	36± 2[b]	153± 9[a]	112± 12[b]	113± 5[b]
Pro	135± 4[a]	64± 6[c]	85± 6[b]	309± 9[a]	163± 5[c]	212± 11[b]
Ser	88± 4	104± 12	97± 7	629± 31[a]	328± 30[c]	406± 15[b]
Taurine	118± 9[a]	72± 2[b]	108± 8[a]	221± 11[a]	79± 5[b]	187± 17[a]
Thr	87± 5[a]	44± 6[c]	57± 4[b]	515± 26[a]	360± 33[b]	358± 20[b]
Trp	37± 2[a]	19± 2[c]	26± 2[b]	71± 4[a]	49± 5[c]	63± 2[b]
Tyr	57± 3[a]	37± 1[b]	42± 3[b]	184± 8[a]	119± 10[b]	163± 12[a]
Val	132± 6[a]	75± 5[b]	87± 5[b]	357± 14[a]	220± 9[b]	254± 12[b]
Total α-AA	2713± 52[a]	1952± 39[c]	2138± 37[b]	6407± 140[a]	4255± 72[c]	4943± 101[b]

Data are means ± SEM. Means with different superscript letters are different ($P < 0.05$). Control, ewes were fed 100% of NRC requirements between Days 28 and 135 of gestation (n = 8). [†]Fed 50% of NRC nutrient requirements betweens Days 28 and 135 of gestation (n = 5). [‡]Fed 50% of NRC nutrient requirements betweens Days 28 and 78 of gestation and realimented to 100% of NRC nutrient requirements between Days 78 and 135 of gestation (n = 8). AA, amino acids.

of gestation are summarized in Table 6.4. Maternal undernutrition between Day 28 and 135 of gestation markedly reduced ($P < 0.05$) concentrations of most amino acids (alanine, arginine, citrulline, cysteine, glutamine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, taurine, threonine, tryptophan, tyrosine, and valine) in both maternal and fetal plasma. Concentrations of β-alanine, aspartate, glutamate, glycine, and serine in maternal plasma, and concentrations of β-alanine and aspartate in fetal plasma did not differ ($P > 0.05$) between control-fed and nutrient-restricted ewes. Realimentation of underfed ewes beginning on Day 78 of gestation increased ($P < 0.05$) concentrations of alanine, cysteine, methionine, proline, taurine, and tryptophan in both maternal and fetal plasma by Day 135 of gestation, compared with ewes fed 50% of NRC nutrient requirements between Days 28 and 135 of gestation. Concentrations of total α-amino acids were 28% and 34% lower ($P < 0.05$), respectively, in maternal and fetal plasma of ewes fed 50% of NRC nutrient requirements between Days 28 and 135 of gestation, when compared with control-fed ewes. Realimentation of underfed ewes completely restored, to values for control-fed ewes, concentrations of alanine in maternal plasma and of alanine, histidine, taurine, and tyrosine in fetal plasma. However, in maternal and fetal plasma, concentrations of most amino acids remained lower ($P < 0.05$) in realimented ewes, in comparison with control-fed ewes. In maternal and fetal plasma, concentrations of total α-amino acids in realimented ewes were 10-16% higher ($P < 0.01$) than those in underfed ewes, but were 21-23% lower ($P < 0.01$) than those in control-fed ewes.

Table 6.5 summarizes concentrations of amino acids in allantoic and amniotic fluids on Day 135 of gestation. Maternal undernutrition between Days 28 and 135 of gestation markedly reduced ($P < 0.05$) concentrations of most amino acids (arginine, citrulline, cysteine, glutamate, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, taurine, tryptophan, tyrosine, and valine) in both allantoic and amniotic fluids. The decrease in allantoic fluid serine (84%) was the most striking. Surprisingly, con-

TABLE 6.5. Amino acid concentrations (μM) in ovine fetal fluids on Day 135 of gestation

Amino acid	Allantoic fluid			Amniotic fluid		
	Control	Restricted[†]	Realimented[‡]	Control	Restricted[†]	Realimented[‡]
Ala	1075± 56[a]	1342± 75[a]	774± 110[b]	194 ± 8[a]	71 ± 2[c]	114 ± 11[b]
β-Ala	1044 ± 49	1162 ± 139	1041 ± 46	162 ± 11	149 ± 8	144 ± 13
Arg	670 ± 44[a]	272 ± 28[c]	476 ± 36[b]	186 ± 8[a]	39 ± 4[c]	95 ± 13[b]
Asn	260 ± 16	274 ± 12	285 ± 17	37 ± 2[a]	17 ± 2[b]	32 ± 3[a]
Asp	182 ± 13	143 ± 11	153 ± 9	22 ± 1[a]	15 ± 2[b]	17 ± 1[b]
Cit	592 ± 35[a]	214 ± 18[c]	318 ± 20[b]	178 ± 8[a]	22 ± 3[c]	43 ± 5[b]
Cys	393 ± 18[a]	196 ± 17[c]	265 ± 15[b]	127 ± 4[a]	50 ± 4[c]	87 ± 5[b]
Gln	1036 ± 50[b]	1469 ± 117[a]	798 ± 74[c]	368 ± 14[a]	86 ± 8[c]	121 ± 8[b]
Glu	215 ± 16[a]	155 ± 10[b]	202 ± 12[a]	69 ± 4[a]	47 ± 3[b]	50 ± 4[b]
Gly	1447 ± 65[b]	3103 ± 373[a]	1299 ± 22[b]	326 ± 14[a]	184 ± 6[c]	240 ± 18[b]
His	341 ± 20[a]	264 ± 12[b]	358 ± 18[a]	26 ± 2[a]	12 ± 1[c]	18 ± 1[b]
Ile	167 ± 8[a]	108 ± 7[b]	103 ± 5[b]	29 ± 2[a]	10 ± 1[c]	14 ± 1[b]
Leu	258 ± 11[a]	151 ± 6[b]	150 ± 7[b]	50 ± 2[a]	17 ± 1[c]	31 ± 2[b]
Lys	493 ± 36[a]	287 ± 17[b]	266 ± 25[b]	55 ± 2[a]	19 ± 2[c]	28 ± 2[b]
Met	197 ± 12[a]	106 ± 4[c]	129 ± 5[b]	22 ± 2[a]	16 ± 1[b]	17 ± 1[b]
Orn	152 ± 11[a]	99 ± 2[b]	89 ± 5[b]	25 ± 1[a]	13 ± 1[b]	14 ± 1[b]
Phe	136 ± 11[a]	105 ± 4[b]	100 ± 6[b]	27 ± 2[a]	9 ± 1[c]	13 ± 1[b]
Pro	590± 26[a]	275± 12[c]	371± 18[b]	141 ± 5[a]	54± 3[c]	82 ± 5[b]
Ser	19814± 556	3110± 157[c]	14438± 1591[b]	771 ± 43[a]	205 ± 4[c]	365 ± 31[b]
Taurine	1010 ± 51	984 ± 69	958 ± 78	127 ± 10	102 ± 8	103 ± 10
Thr	494 ± 28[b]	1087 ± 85[a]	486 ± 83[b]	201 ± 13[a]	64 ± 4[c]	99 ± 6[b]
Trp	165 ± 7[a]	86 ± 3[c]	112 ± 6[b]	15 ± 1[a]	9 ± 1[b]	13 ± 1[a]
Tyr	222 ± 10[a]	180 ± 7[b]	182 ± 18[b]	44 ± 2[a]	13 ± 1[c]	20 ± 1[b]
Val	407 ± 16[a]	229 ± 15[c]	304 ± 9[b]	78 ± 4[a]	28 ± 2[c]	50 ± 4[b]
Total α-AA	29304 ± 615	13255 ± 373[c]	23168 ± 526[b]	2988 ± 86[a]	998 ± 22[c]	1620 ± 70[b]

Data are means ± SEM. Means with different superscript letters are different (P < 0.05). Control, ewes were fed 100% of NRC requirements between Days 28 and 135 of gestation (n = 8). [†]Fed 50% of NRC nutrient requirements betweens Days 28 and 135 of gestation (n = 5). [‡]Fed 50% of NRC nutrient requirements betweens Days 28 and 78 of gestation and realimented to 100% of NRC nutrient requirements between Days 78 and 135 of gestation (n = 8). AA, amino acids.

centrations of alanine, glutamine, glycine, and threonine in allantoic fluid increased (P < 0.01) in underfed ewes compared with control-fed ewes. Concentrations of alanine, β-alanine, asparagine, and taurine in allantoic fluid or concentrations of β-alanine and aspartate in amniotic fluid did not differ (P > 0.05) between control-fed and nutrient-restricted ewes. Concentrations of total α-amino acids were 55% and 67% lower (P < 0.01), respectively, in allantoic and amniotic fluids of underfed ewes, when compared with control-fed ewes. Realimentation of underfed ewes beginning on Day 78 of gestation increased (P < 0.05) concentrations of arginine, citrulline, cysteine, histidine, proline, serine, tryptophan, and valine in both allantoic and amniotic fluids, compared with non-realimented ewes. However, in these fetal fluids, concentrations of most amino acids in allantoic and amniotic fluids remained lower (P < 0.05) in realimented ewes, in comparison with control-fed ewes. Realimentation of underfed ewes completely restored, to values for control-fed ewes, concentrations of glutamate

TABLE 6.6. Polyamine concentrations (μM) in ovine fetal fluids on Day 135 of gestation

Polyamine	Allantoic fluid			Amniotic fluid		
	Control	Restricted[†]	Realimented[‡]	Control	Restricted[†]	Realimented[‡]
Putrescine	423 ± 17^a	152 ± 16^c	267 ± 17^b	396 ± 18^a	131 ± 10^c	273 ± 11^b
Spemidine	723 ± 28^a	236 ± 21^c	517 ± 20^b	589 ± 21^a	164 ± 12^c	383 ± 25^b
Spermine	946 ± 66^a	303 ± 23^c	647 ± 31^b	2787 ± 76^a	821 ± 43^c	1697 ± 93^b
Total PA	2092 ± 38^a	691 ± 42^c	1431 ± 38^b	3773 ± 84^a	1116 ± 52^c	2353 ± 78^b

Data are means ± SEM. Means with different superscript letters are different (P < 0.05). Control, ewes were fed 100% of NRC requirements between Days 28 and 135 of gestation (n = 8). [†]Fed 50% of NRC nutrient requirements betweens Days 28 and 135 of gestation (n = 5). [‡]Fed 50% of NRC nutrient requirements betweens Days 28 and 78 of gestation and realimented to 100% of NRC nutrient requirements between Days 78 and 135 of gestation (n = 8). PA, polyamines.

and histidine in allantoic fluid and of asparagine and tryptophan in amniotic fluid. In both allantoic and amniotic fluids, concentrations of total α-amino acids (Table 6.5) and polyamines (Table 6.6) in realimented ewes were higher (P < 0.01) than those in underfed ewes, but were lower (P < 0.01) than those in control-fed ewes.

Discussion

There is growing interest in the Barker hypothesis of the "fetal origin of adult disease" initially formulated on the basis of human epidemiological studies of the offspring exposed to undernutrition *in utero* (Barker and Clark, 1997). Elucidation of the underlying mechanisms will improve strategies to ensure optimal fetal growth and development, as well as reduce the risk of chronic diseases in adult life. Despite reports of reduced concentrations of amino acids in plasma of fetal rats (Malandro et al., 1996) as well as plasma and fluids of fetal pigs (Wu et al., 1998a) in response to maternal protein deficiency, little else is known about the effects of undernutrition on the concentrations of amino acids and polyamines in the conceptus of any species. In addition, although changes in concentrations of amino acids in maternal and fetal plasma of women with IUGR were documented (Cetin et al., 1988, 1990, 1992a, 1996; Economides et al., 1989), whether these changes were caused by maternal undernutrition or other genetic and environmental factors was not known.

This is the first report of changes in concentrations of both amino acids and polyamines in the ovine conceptus in response to experimentally controlled maternal undernutrition. There are four major findings from this study: 1) maternal undernutrition markedly reduced concentrations of arginine-family amino acids (arginine, citrulline, glutamine, ornithine and proline) and branched-chain amino acids in maternal and fetal plasma and in fetal fluids; 2) serine exhibited the most striking decrease in allantoic fluid (84%) and amniotic fluid (73%) by late gestation; 3) concentrations of polyamines in allantoic and amniotic fluids were much lower in underfed ewes than in control-fed ewes; and

4) realimentation of underfed ewes increased concentrations of total α-amino acids in maternal and fetal plasma and of total α-amino acids (particularly serine) and polyamines in fetal fluids, in association with the compensatory growth of the fetal lambs.

An important finding of this study was that concentrations of all essential amino acids in maternal plasma (except for histidine on Day 78 of gestation) decreased markedly at both mid- and late-gestation in response to nutrient restriction (Tables 6.1 and 6.4). This result is in sharp contrast to the report that concentrations of most essential amino acids in maternal plasma were elevated in women with IUGR of unknown etiology (Cetin et al., 1996). Plasma concentrations of amino acids in pregnant animals depend on several factors, including rate of amino acids entering the portal vein, rates of whole body amino acid synthesis and catabolism, rate of amino acid transfer from maternal to fetal plasma, and whole body protein turnover (synthesis and degradation). Because of reduced dietary intake of protein, the entry of amino acids to the portal vein and the synthesis of nonessential amino acids were lower in nutrient-restricted ewes, compared with control-fed ewes. A decrease in concentration of urea (a major nitrogenous product of protein catabolism) in maternal and fetal plasma of underfed ewes (Vonnahme et al., 2003) suggests a decrease in amino acid degradation by the whole body. Likewise, owing to reduced placental-fetal blood flows (Ford, 1995), transfer of amino acids from maternal to fetal plasma would be reduced in nutrient-restricted ewes, compared with control-fed ewes. Thus, the mobilization of maternal protein reserve (mainly from skeletal muscle) did not appear to sufficiently compensate for the decrease in dietary protein intake. Our finding that concentrations of total α-amino acids in both maternal and fetal plasma were decreased in underfed ewes supports the view that placental transport is a major mechanism responsible for amino acid homeostasis in the fetus (Bell and Ehrhardt, 2002).

Each amino acid has its own unique metabolic pathway that is cell- and tissue-specific manner (Stipanuk and Watford, 2000). Consistent with this

notion, maternal undernutrition differentially affected concentrations of amino acids in maternal and fetal plasma (Tables 6.1 and 6.4). For example, on Day 78 of gestation, concentrations of glycine in both maternal and fetal plasma of nutrient-restricted ewes increased 47-55%, but concentrations of most amino acids (including serine) decreased, when compared with control-fed ewes (Table 6.1). In contrast, on Day 135 of gestation, concentrations of glycine and serine in maternal plasma were similar between control-fed and nutrient-restricted ewes, but their concentrations in fetal plasma decreased by 40-48% in comparison with control-fed ewes (Table 6.1). Our results indicate the metabolism of different amino acids varies with maternal undernutrition. Although the alterations in amino acid metabolism may be beneficial for fetal survival and growth in response to maternal undernutrition, they may have an important impact on postnatal life.

Remarkably, concentrations of arginine-family amino acids (arginine, citrulline, and proline) and all branched-chain amino acids (isoleucine, leucine and valine) consistently decreased in all measured compartments of underfed ewes at mid- and late gestation. Similarly, IUGR in humans is associated with reduced concentrations of arginine (Bajoria et al., 2001) and branched-chain amino acids (Cetin et al., 1988, 1990, 1992a, 1996) in fetal plasma. A decrease in arginine availability would reduce endothelial nitric oxide synthesis (Wu and Meininger, 2002), and, therefore, placental-fetal blood flows. Furthermore, a reduced availability of branched-chain amino acids in maternal and fetal plasma would impair the synthesis of glutamine by placenta and fetal tissues (e.g., skeletal muscle), which is a major fuel for the growing fetus, a primary transporter of both carbon and nitrogen among fetal organs, and an essential substrate for the synthesis of DNA and aminosugars (Flynn et al., 2002).

Allantoic fluid is derived from fetal and maternal secretions, but primarily from placental transport mechanisms (Bazer, 1989). Although early anatomical studies suggested that the allantoic sac served as a reservoir for fetal wastes, it is now clear that amino acids and their metabolic products (e.g., polyamines) in

allantoic fluids may be absorbed by the allantoic epithelium into the fetal-placental circulation and utilized by fetal-placental tissues (Bazer, 1989; Kwon et al., 2003a, 2003b). We recently reported an unusual abundance of serine (16–19 mM) in ovine allantoic fluid during late gestation (Days 100-140) (Kwon et al., 2003a), suggesting a critical role for this neutral amino acid in fetal growth. Strikingly, serine concentration in allantoic fluid decreased 84% by Day 135 of gestation in underfed ewes, compared with control-fed ewes (Table 6.4).

Our finding that serine was the only amino acid that exhibited such a marked change in response to maternal undernutrition raised an important question regarding serine metabolism and its significance to fetal development. The available evidence indicates that there is uterine uptake of serine from maternal plasma, but there is little transplacental transport of serine to the ovine fetus owing to its extensive catabolism by uteroplacental tissues (Moores et al., 1994; Bell et al., 1989). Thus, in fetal lambs, large amounts of serine are synthesized from glycine and N^5,N^{10}-methylenetetrahydrofolate via serine hydroxymethyl-transferase and from 3-phosphoglycerate (an intermediate of glycolysis) and glutamate via phosphoglycerate dehydrogenase and phosphoserine aminotransferase (Moores et al., 1994; Narkewicz et al., 1999). All these enzymes are present in fetal ovine liver and kidney, with the liver being the major organ for serine synthesis in fetal sheep (Narkewicz et al., 1999, 2002). The reduction in serine seen as a result of maternal undernutrition may therefore be an indicator of liver and/or kidney damage, both of which have long term metabolic and cardiovascular consequences later in life. Maternal undernutrition may also reduce the expression of these enzymes, thereby suppressing serine production. In support of this view, the concentration of glycine in allantoic fluid on Day 135 of gestation was markedly elevated in nutrient-restricted ewes (Table 6.5). Serine is a major glucogenic amino acid in humans and ewes (Clark et al., 1976). It also plays an important role in one-carbon unit metabolism essential for 2'-deoxythymidylate synthesis and methylation (Snell and Fell, 1990). In addition, serine participates in the

synthesis of phosphatidylserine and ceramide (signaling molecules) (Stipanuk and Watford, 2000). All of these events are critical for DNA synthesis and thus cell proliferation. Therefore, we surmise that a reduced availability of serine in the conceptus may impair the synthesis of glucose, DNA and protein, thereby contributing to IUGR in underfed ewes.

Amniotic fluid is composed of water and electrolytes from both the fetus (kidneys, lungs, epidermis, and fetal blood vessels in the placenta and umbilical cord) and the mother (decidual blood vessels via amniotic membranes) (Schmidt, 1992). This fluid is removed by both the fetus and the mother through the same channels, along with the participation of the fetal intestine following swallowing (Schmidt, 1992). Through the kidney, bladder and urachus, and placental vasculature, there are nutrient exchanges between amniotic and allantoic fluid (Bazer, 1989). We recently reported that glutamine (a major fuel for enterocytes and a regulator of intestinal function) and polyamines (essential for intestinal cell proliferation and differentiation) are particularly abundant in amniotic and allantoic fluids (Kwon et al., 2003a, 2003b). Thus, with the development of intestinal amino acid and polyamine transport systems during gestation (Sagawa et al., 1979), the drinking of amniotic fluid provides a source of both amino acids and polyamines for utilization by the fetal intestine and other fetal tissues. The nutritional significance of amniotic fluid is graphically illustrated by the finding that esophageal ligation, which prevents the entry of this fluid into the small intestine, results in IUGR in fetal sheep (Trahair and Harding, 1995). Remarkably, concentrations of both glutamine and polyamines were severely depleted in ovine amniotic and allantoic fluids at mid- and late gestation (Tables 6.3 and 6.6). The reduced availability of glutamine and polyamines may contribute to the retarded growth of the fetal small intestine and other fetal tissues in underfed ewes.

Realimentation of underfed ewes beginning from mid-gestation increased concentrations of total α-amino acids in maternal and fetal plasma as well as α-amino acids and polyamines allantoic and amniotic fluids, compared with non-

alimented ewes (Table 6.4). These results indicate that increasing maternal concentrations of amino acids can increase their availability in fetal plasma and fluids. Importantly, realimentation of underfed ewes increased serine concentrations in allantoic fluid 4-fold in comparison with underfed ewes (Table 6.5), suggesting an increase in intra-fetal synthesis of serine. However, in all of the measured compartments, concentrations of total α-amino acids and polyamines remained lower in realimented ewes, compared with control-fed ewes. There are several possible explanations for these findings. First, utilization of amino acids for tissue protein synthesis was augmented to meet the increased need for compensatory growth of both the mother and the fetus between Days 78 and 135 of gestation. Because the provision of dietary protein to realimented ewes was the same for control-fed ewes (per kg metabolic weight), an increase in tissue protein synthesis in both dams and fetal lambs likely led to decreased availability of free amino acids in maternal and fetal plasma and in fetal fluids. Second, because underfed ewes exhibit compensatory growth of placentomes in response to realimentation during late gestation (Gardner et al., 2002), increased amounts of amino acids would be directed towards placentomal protein synthesis, thereby reducing their transfer from maternal and fetal blood. Third, arginine, ornithine and methionine, as well as proline and glutamine (precursors of ornithine) are important substrates for the synthesis of putrescine, spermidine and spermine (Wu and Morris, 1998), and, therefore, the reduced availability of these amino acids in fetal plasma and fluids would contribute to a decrease in polyamine synthesis by placental, uterine and fetal tissues.

There are multiple genetic and environmental factors that can cause IUGR (Marsal, 1995). Although the fetal genome plays an important role in growth potential *in utero*, increasing evidence suggests that the intrauterine environment is a major determinant of fetal growth. For example, embryo-transfer studies show that it is the recipient mother rather than the donor mother that more strongly influences fetal growth (Brooks et al., 1995). Among

intrauterine environmental factors, nutrition appears to play a critical role in influencing placental growth and fetal programming (Godfrey and Robinson, 1998). Because amino acids and their metabolic products (e.g., nitric oxide and polyamines) are crucial for placental-fetal growth and development (Sladek et al., 1997; Wu and Morris, 1998; Reynolds and Redmer, 2001; Bird et al., 2003), increasing provision of arginine, serine, branched-chain amino acids, and other amino acids may help prevent IUGR. In humans, even a modest increase in fetal growth will bring a significant improvement in pregnancy outcomes (Brooks et al., 1995; Godfrey and Robinson, 1998; Marsal, 2002; Kramer, 2003). Therefore, identifying new means to promote an optimal intrauterine environment will be very beneficial to ensure optimal pregnancy outcomes while saving billions of healthcare dollars annually both in the U.S. and around the world.

In conclusion, the availability of total α-amino acids, particularly serine, arginine-family amino acids, and branched-chain amino acids, were severely reduced in maternal and fetal plasma and in fetal fluids of underfed ewes. Maternal undernutrition also markedly decreased polyamine concentrations in fetal allantoic and amniotic fluids. Realimentation of underfed ewes beginning from mid-gestation increased concentrations of total α-amino acids in maternal and fetal plasma and of polyamines in fetal fluids. These findings establish a foundation for further studies to define the roles of amino acids and polyamines in the prevention and treatment of IUGR brought about by maternal undernutrition.

CHAPTER VII
SUMMARY AND CONCLUSIONS

This is the first known report of changes in concentrations of amino acids in ovine fetal fluids and plasma, and changes in polyamine synthesis and concentrations, and NO synthesis in ovine placental and endometrial tissues throughout gestation. Four major findings from these studies were: (1) concentrations of amino acids and polyamines in ovine allantoic and amniotic fluids, plasma and utero-placental tissues change markedly during gestation; (2) the activities of ODC, arginase, and NO synthase are expressed in ovine placentomes, intercotyledonary placenta, and intercaruncular endometrium; (3) polyamine synthesis and NO production vary greatly among ovine placental and endometrial tissues, fetal fluids and with day of gestation; and (4) maternal undernutrition markedly reduced concentrations of amino acids and polyamines in fetal fluids.

The experiment described in Chapter III was conducted to test the hypothesis that arginine is the most abundant amino acid in ovine allantoic fluid during early gestation, because an unusual abundance of arginine (4 to 6 mM) was previously reported for porcine allantoic fluid during early gestation (Wu et al., 1996). However, it is not known whether such high concentrations of arginine are unique for porcine allantoic fluid or if they represent an important physiological phenomenon for mammals. The measurement of amino acids other than arginine is necessary to determine its relative abundance in ovine fetal fluids. In contrast to high concentrations of arginine in porcine allantoic fluid during early gestation, arginine was not the major amino acid in ovine allantoic fluid during early gestation. Rather, ovine allantoic fluid was particularly rich in four of the traditionally classified nonessential amino acids: alanine, citrulline, glutamine, and serine (Table 3.7). Concentrations of alanine, citrulline, and glutamine in allantoic fluid increased by 20-, 34-, and 18-fold, respectively, between Days 30 and 60 of gestation, and were 24.7, 9.7, and 23.5 mM,

respectively, on Day 60 of gestation (compared with 0.8 mM arginine). Remarkably, alanine, citrulline and glutamine accounted for about 80% of total α-amino acids in allantoic fluid during early gestation. Serine (16.5 mM) contributed about 60% of total α-amino acids in allantoic fluid on Day 140 of gestation. The unusual abundance of alanine and citrulline on Day 60 of gestation and of serine during late gestation has not been reported for any other biological fluid in animals (e.g., Lemons et al., 1976; Marconi et al., 1989; Snell and Fell, 1990; Curthoys and Watford, 1995; Wu et al., 1996; Wu and Morris, 1998; Stipanuk and Watford, 2000). However, the increases in concentrations of total amino acids in allantoic fluids during early pregnancy could not be accounted for by changes in allantoic fluid volume (Table 3.1) or by changes in concentrations of amino acids in maternal and fetal plasma (Tables 3.2 and 3.3).

On the other hand, alanine, glutamine, glycine and serine in amniotic fluid were the most abundant α-amino acids at all gestational ages and contributed approximately 50 % of total α-amino acids. Glycine was the most abundant amino acid in maternal uterine arterial plasma, representing about 25% of total α-amino acids. Alanine, glutamine, glycine and serine contributed about 50% of total α-amino acids in fetal plasma. Fetal:maternal plasma ratios for amino acids varied greatly, being < 1 for glutamate during late gestation, 1.5 to 3 for most amino acids throughout gestation, and > 10 for serine during late gestation.

Results of this study raise important questions regarding the origin and function of alanine, glutamine, citrulline, and serine in ovine fetal-placental nutrition and metabolism. Both glutamine and alanine may be synthesized from branched-chain amino acids in fetal ovine skeletal muscle (Goodwin et al., 1987). On the basis of maternal arterial-fetal umbilical venous differences in amino acid concentrations (Tables 3.2 and 3.3), it is suggested that the ovine placenta synthesizes citrulline. Arginine is the most abundant nitrogen carrier in tissue proteins (Meier et al., 1981; Wu et al., 1999a). Whatever the differences among species, the unusual abundance of either citrulline (an effective precursor of arginine) in ovine allantoic fluid (Table 3.7) or arginine in porcine

allantoic fluid (Wu et al., 1995a, 1996a, 1998a) during early gestation raises intriguing and important questions regarding the physiological significance of arginine-dependent pathways in fetal-placental nutrition and development. It is now clear that allantoic fluid nutrients may be absorbed by the allantoic epithelium into the fetal-placental circulation and utilized by fetal-placental tissues (Bazer, 1989), although early anatomical studies suggested that the allantoic sac served only as a reservoir for fetal wastes. The amino acids that accumulate in allantoic fluid during early gestation may play an important role in fetal growth and development.

The experiment described in Chapter IV was designed to determine the ODC and arginase activities, arginine, ornithine, and polyamine concentrations, and polyamine synthesis during pregnancy, because marked increases in concentrations of both ornithine and arginine (substrate for polyamine synthesis) in ovine allantoic fluids (a reservoir for nutrients in the fetus) between Days 30 and 60 of gestation, and amniotic and allantoic derive, in part, from secretions and transport of water across the placenta and endometrium. Ovine allantoic fluid is rich in ornithine and arginine (Kwon et al., 2003a), and is likely a major source of these two amino acids for placenta and endometrium. This view is supported by the fact that the highest concentrations of arginine in ovine allantoic fluid on Day 100 of gestation (Kwon et al., 2003a) were associated with the highest concentrations of arginine in intercotyledonary placenta and intercaruncular endometrium (Table 4.7) and with elevated concentrations of arginine in placentomes (Table 4.7). In addition, between Days 30 and 80 of gestation, increases in substrate concentrations and polyamine synthesis in ovine placental and endometrial tissues (Tables 4.4-4.6) are associated with increases in ornithine and arginine concentrations in ovine allantoic fluid (Kwon et al., 2003a), further supporting the earlier suggestion that this fetal fluid is an important nutrient reservoir during gestation (Bazer, 1989). In ovine allantoic and aminotic fluids, polyamines were most abundant during early (Days 40-60) and late (Days 100-140) gestation. Increases in polyamine concentrations in

allantoic fluid between Days 30 and 60 of gestation (Table 4.8) were closely correlated with increases in polyamine synthesis in ovine placentomes and endometrium (Tables 4.4 and 4.5).

Polyamine synthesis is regulated by ODC and arginase activities as well as the availability of ornithine and arginine (Wu and Morris, 1998; Li et al., 2001; Wei et al., 2001), because, through the formation of ornithine, arginase would be expected to play a regulatory role in polyamine synthesis from arginine and arginine was actively converted into polyamines in ovine placental and endometrial tissues (Table 4.5). Thus, rates of polyamine synthesis were closely correlated with arginase activity in these tissues (Table 4.2) and ODC activity was positively correlated with polyamine concentrations in placentomes and endometrium (Table 4.11). In addition, concentrations of both ornithine and polyamines peaked between Days 60 and 80 of gestation in intercotyledonary placenta (Tables 4.5 and 4.6). These results further support an important role for polyamine synthesis in regulating polyamine concentrations.

Collectively, placentomal arginase activity and arginine concentrations peaked on Day 40 of gestation (Table 4.2), thus maximizing the hydrolysis of arginine to ornithine and markedly increasing intracellular concentrations of ornithine (Table 4.7). The latter was coupled with the highest ODC activity for polyamine synthesis (Table 4.1), thereby maximizing polyamine concentrations (Table 4.6) in ovine placentomes during early gestation. Similarly, arginase and ODC activities, intracellular ornithine concentrations, and polyamine synthesis from both ornithine or arginine were maximal in intercaruncular endometrium on Day 40 of gestation, resulting in highest concentrations of polyamines (Table 4.6). Furthermore, concentrations of glutamine, a major substrate for ornithine and arginine synthesis (Wu and Morris, 1998) and a stimulator of ODC activity (Wu et al., 2000c), were highest in ovine allantoic fluid between Days 40 and 60 of gestation (Kwon et al., 2003a).

Finally, arginine is an important precursor for polyamine synthesis in the ovine placenta and endometrium, metabolic coordination among the several

integrated pathways that support high rates of polyamine synthesis in the placenta and endometrium during early gestation.

The experiment described in Chapter V was designed to determine the NOS and GTP-CH activities, NO synthesis, tetrahydrobiopterin and NADPH in placenta and endometium, because arginase and NOS use arginine as a common substrate, and metabolic coordination among the several integrated pathways that may support high rates of NO in placenta and endometrial tissues. Placental synthesis of NO, like that of polyamines (other products of arginine catabolism essential for placental angiogenesis and growth), increased markedly between Days 30 and 60 of gestation when placental growth and placentomal development are most rapid.   Likewise, NO synthesis in intercaruncular endometrium peaked on Days 40-60 of gestation, when this tissue undergoes marked morphological and functional changes. 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 (Ford, 1995; Reynolds and Redmer, 1995).   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. In addition, 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 intercotylednonary placenta, cNOS activity was less and greater than iNOS activity on Days 30 and 80 of gestation, respectively.

Placentomal GTP-CH activity peaked on Day 60 of gestation (Table 5.2), thus maximizing the *de novo* synthesis of BH4. Consequently, placentomal BH4 and NADPH concentrations were highest on Day 60 of gestation (Table 5.5). All of these changes contribute to maximal NO production during early pregnancy.

Thus, 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. 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. In addition, 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 5.4), as did allantoic fluid concentrations of citrulline (the precursor of arginine) and arginine (Kwon et al., 2003a). Between Days 80 and 100 of gestation, BH4 concentrations also increased in placentome and endometrium (Table 5.4), as did concentrations of arginine in allantoic fluid (Kwon et al., 2003a). 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 5.5).

Finally, 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 flows increase 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.

These novel results may have important implications for both intrauterine growth retardation and fetal origins of diseases in adults, because recent epidemiological studies in humans suggest links between intrauterine growth retardation and development of chronic disease (e.g., diabetes, hypertension, and coronary heart disease) later in life (Barker and Clark, 1997; Symonds et al., 2001).

The experiment described in Chapter VI was designed to determine the concentrations of amino acids and polyamines in fetal fluids in response to

maternal undernutrition, because the significance of these changes for normal fetal growth and development may be underscored under a pathophysiological condition. Concentrations of all essential amino acids in maternal plasma (except for histidine on Day 78 of gestation) decreased markedly at both mid- and late-gestation in response to nutrient restriction (Tables 6.1 and 6.4). In addition, concentrations of arginine-family amino acids (arginine, citrulline, and proline) and all branched-chain amino acids (isoleucine, leucine and valine) consistently decreased in all measured compartments (including maternal and fetal plasma as well as allantoic and amniotic fluids) of underfed ewes at mid- and late gestation. Particularly, serine exhibited the most striking decrease in allantoic fluid (84%) and amniotic fluid (73%) by late gestation in response to maternal undernutrition. On the other hand, realimentation of underfed ewes increased concentrations of total α-amino acids in maternal and fetal plasma, and concentrations of polyamines and total α-amino acids (particularly serine in allantoic fluid by 4-fold over value for underfed ewes) (Table 6.5) in fetal fluids, because of suggesting an increase in intra-fetal synthesis of serine. However, in all of the measured compartments, concentrations of total α-amino acids and polyamines remained lower in realimented ewes, compared with control-fed ewes. Concentrations of polyamines in allantoic and amniotic fluids were much lower in underfed ewes than in control-fed ewes.

In conclusion, remarkable changes occur in the concentrations of amino acids in ovine fetal allantoic fluid between Days 30 and 140 of gestation. In this fluid, alanine, citrulline, and glutamine contributed approximately 80 % of total α-amino acids during early gestation, and serine accounted for approximately 60 % of total α- amino acids during late gestation. In addition, polyamine synthesis and concentrations, and NO synthesis were highest in both placentomes and endometrium of ewes on Days 40, and 60 of gestation (respectively) when their growth and morphological changes are most rapid and when fetal-placental blood flows increase substantially. However, concentrations of amino acids and polyamines in fetal fluids markedly decreased

in response to maternal undernutrition. Therefore, amino acids and their metabolic products (e.g., nitric oxide and polyamines) are crucial for placental-fetal growth and development (Sladek et al., 1997; Wu and Morris, 1998; Reynolds and Redmer, 2001; Bird et al., 2003), and, because of increasing provision of arginine, serine, branched-chain amino acids, and other amino acids they may help prevent IUGR. These results establish a new base of information for future studies to define the roles of amino acids and polyamines in fetal-placental growth and development. Future studies are necessary to detect mRNAs and proteins for NO synthases, ODC using *in situ* hybridization and immunohistochemical analysis.

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